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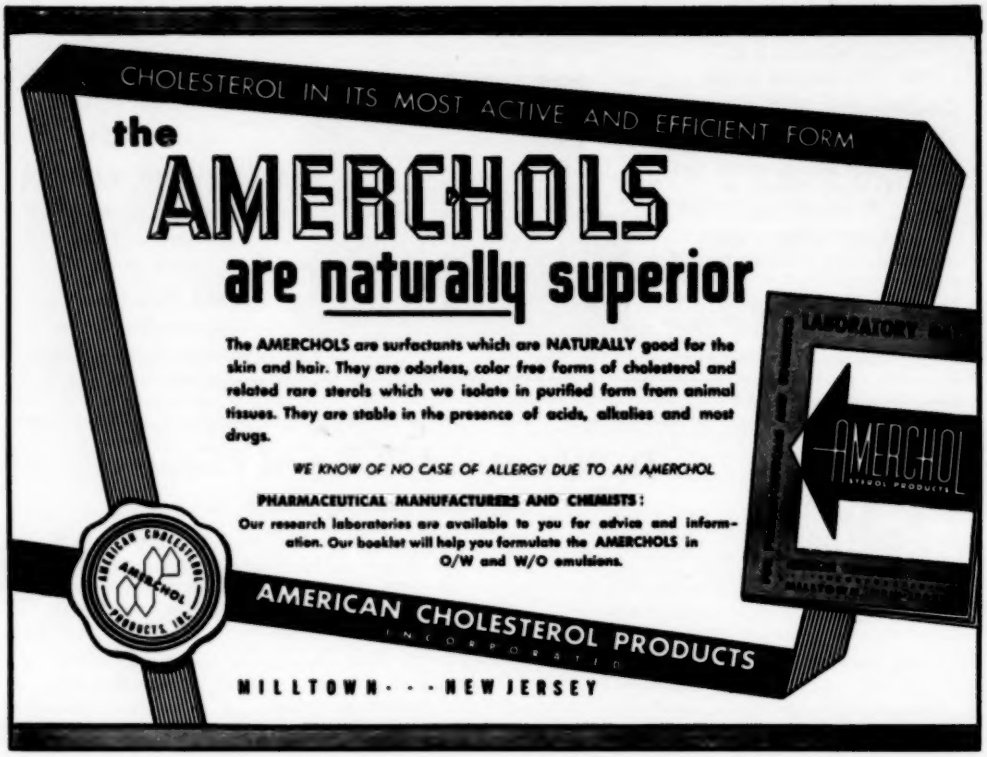
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*Scientific Edition*

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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## Stability of Vitamin $B_{12}$—Folic Acid Parenteral Solutions*

By ABRAHAM TAUB† and HERBERT LIEBERMAN‡

**The low solubility of folic acid in water, except at a relatively high *p*H, has prevented its use in parenteral solutions in combination with vitamin $B_{12}$ (cyanocobalamin), which attains optimum stability in the *p*H range 4 to 6.5. The present study utilizes selected solubilizers for folic acid, including aminoacetic acid, methyl glucamine, and niacinamide within a *p*H range of 6 to 6.5. Results indicate that niacinamide is the best of the solubilizers studied. By special processing it is possible to dissolve and maintain in solution 5 mg. of folic acid per cc. at *p*H 6 under conditions compatible with crystalline vitamin $B_{12}$ at a concentration of 30 μg. per cc. Solutions show no significant loss of potency of either the folic acid or the vitamin $B_{12}$ after storage at room temperature and under accelerated conditions, and display satisfactory stability with respect to clarity of solution.**

VITAMIN $B_{12}$ and folic acid are closely related in fundamental biochemical processes. The observation that folic acid is a potentiator of vitamin $B_{12}$ in the rapid regeneration of hemoglobin (1) and the prevailing belief that both factors are required for the satisfactory management of several types of macrocytic anemias (2) have made it desirable to provide both factors in a single dosage form. Although this is readily possible when both are admixed in the solid dosage forms, the preparation of liquid vehicles, particularly parenteral solutions containing relatively high concentrations, is limited by the marked instability of vitamin $B_{12}$ at the high *p*H levels necessary for suitable folic acid concentrations and the rather slight solubility and poor stability of folic acid at the low *p*H levels compatible with vitamin $B_{12}$ stability.

Folic acid is very slightly soluble in water at room temperature [approximately 0.004 mg. per cc. (*p*H 3)]; its solubility increases with rise in *p*H. Biamonte and Schneller (3), studying the effect of *p*H on folic acid solubility by use of buffer solutions, found the solubility of folic acid to be about 0.006 mg. per cc. at *p*H 4, 0.05 mg. per cc. at *p*H 5, and 2 mg. per cc. at *p*H 6. At *p*H 7 the solubility is 15 mg. per cc. and increases rapidly as alkali folate is formed. These authors also showed that below *p*H 6, there was marked decomposition of dissolved folic acid. The loss was 43 per cent at *p*H 5 and 100 per cent at *p*H 4 or below after eleven months' storage at room temperature. At *p*H 6 the loss was insignificant. Biamonte and Schneller also studied the influence on folic acid stability of other B vitamins in various vehicles but did not include vitamin $B_{12}$ in their experiments. They worked at the

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relatively low level of 1 mg. of folic acid per cc. No studies on the stability of higher concentrations of folic acid in pharmaceutical liquids in the acid range appear in the literature.

Crystalline vitamin $B_{12}$ (cyanocobalamin) is soluble to the extent of 12 mg. per cc. in water at 25°; the solution is neutral. The optimum *p*H range of stability at room temperature for aqueous parenteral solutions of crystalline vitamin $B_{12}$ has been reported as 4.5 to 6.5 by Hartley, *et al.* (4). Frost, *et al.* (5), in comparing the stability of analogs of cobalamin, in accelerated tests of three hours at 99°, found that approximately the above *p*H range was optimal for crystalline vitamin $B_{12}$, and that no significant loss occurred between *p*H 4 and about *p*H 6.5. However, they reported that vitamin $B_{12b}$ decomposed appreciably in this test and that the loss increased progressively from 30 per cent at *p*H 2.6 to 90 per cent at *p*H 8.

Thus the limiting *p*H range for maintaining stable solutions of folic acid and vitamin $B_{12}$, assuming the two factors to be otherwise compatible, is 6.0 to 6.5, preferably near *p*H 6.0. Since a folic acid concentration of not less than 5 mg. per cc. is desirable in a parenteral dosage form, the problem was to find a suitable solubilizer for folic acid, and to determine whether such solubilizer, together with folic acid and vitamin $B_{12}$, will remain stable in solution for a suitable storage period.

No studies have been reported on combinations of folic acid and vitamin $B_{12}$ in parenteral solution. A few products containing such combinations have appeared on the market, but analysis of several has shown that their stability with respect to both vitamin $B_{12}$ and folic acid is poor. In some instances, liver injection has been used as a vehicle. A report by Klein (6) has indicated the presence in liver injections of natural solubilizing ingredients that dissolve folic acid and appear to have a stabilizing effect. At the time of the present experiment, however, no quantitative data have been published concerning the degree of solubilization, the extent of the stabilizing influence, or the optimum *p*H for such combinations.

## EXPERIMENTAL

In searching for potential folic acid solubilizing agents, it was found early in the screening process that better results were obtained if the folic acid was first dissolved in dilute sodium hydroxide solution at *p*H 8.5, and dilute hydrochloric acid was then carefully added to *p*H 6.0. More folic acid (approximately 5 mg./cc.) thus remained in solution than if it had been suspended in water and the *p*H adjusted directly to 6 with dilute sodium hydroxide solution. The latter procedure produced folic acid solutions of only about 2 mg./cc.

When the former process was carried out in the absence of solubilizers, the clear solution of 5 mg./cc. of folic acid tended to cloud after storage periods of several days to several weeks. However, in the presence of certain agents, which were added before the solution was acidified to *p*H 6, the solutions remained clear for relatively long periods. In the presence of some solubilizing agents (aminoacetic acid and niacinamide), it was found possible to dissolve the folic acid in 10% solutions of the solubilizers at 75° by bringing the *p*H up to *p*H 6 directly with sodium hydroxide solution. The folic acid was maintained at a concentration of 5 mg./cc. upon cooling to 25°.

Neither the literature nor the chemical structure of folic acid provided any guide for the selection of solubilizing agents. Pharmaceutical experience led to the choice of a small group of substances for experimentation. In general the procedure followed was to dissolve the folic acid in 0.1 *N* NaOH and adjust the *p*H to 8.5; the solubilizing agents were then added in amounts usually representing 0.5 to 10% of the final solution, and the *p*H of the solutions were then adjusted to levels from 5.5 to 6.5 by the careful addition of 0.1 *N* HCl. The concentration of folic acid in the finished solutions was 5 mg./cc. in each case. When the basic agents ethylene diamine and methyl glucamine were used, the folic acid was brought into solution to *p*H 8.5 without the use of sodium hydroxide.

All solutions were examined for clarity after one hour, twenty-four hours, and one week. Those that did not remain clear for a week were eliminated from further consideration. The following agents did not prove suitable as solubilizers: citric acid-sodium citrate buffer, gluconic acid, glutamic acid, sodium benzoate, sodium gentisate, ethylene diamine, triethanolamine, urea, lysine, polyoxyethylene 20 sorbitan monooleate, and propylene glycol (up to 75% concentration).

The folic acid solutions of the following compounds remained clear in the *p*H range 6.0 to 6.5 for more than one week: methyl glucamine, 1%; aminoacetic acid (glycine), 10%; and niacinamide, 10%. Niacinamide was found to have the best solubilizing action; it maintained a concentration of 5 mg. of folic acid per cc. in solution down to a *p*H of 5.6 when prepared by the procedure described. However, solutions were not prepared at *p*H levels below 6 because of the poor stability of the folic acid below that value.

The three potential solubilizers were selected for further stability tests in solutions containing both vitamin $B_{12}$ and folic acid. In addition, dextrose was added to the series of stability tests because the stabilizing effect of sugars on folic acid had been previously reported (7). Vitamin $B_{12}$ and folic acid controls were also set up without stabilizers or solubilizers, using dilute sodium hydroxide solution followed by dilute hydrochloric acid to attain clear solutions at *p*H levels of 6 and 6.5.

All solutions were prepared aseptically with water for injection in which 0.5% of redistilled phenol, of a grade previously found suitable for vitamin $B_{12}$ parenteral solutions, was dissolved as bacteriostatic agent. The vitamin $B_{12}$ used was crystalline ampul grade (Merck), and the folic acid was U. S. P.

grade (Calco). The moisture content of each was determined and solutions calculated on an anhydrous basis were made at final concentrations of 15 μg. of vitamin $B_{12}$ and 5 mg./cc. of folic acid.

The solutions were first passed through filter paper and then filtered through fine-fritted Pyrex filters which had been washed and sterilized. The solutions were then filled into 5-cc. Kimble Neutraglass vials, and sealed with West No. 124 pink rubber stoppers. The stoppers and vials were thoroughly washed and sterilized in accordance with good standard manufacturing practice. Solutions were protected from bright diffused light, and aseptic precautions were maintained throughout the several steps of manufacturing, filtering, filling, and sealing. Samples of end products were found to meet the U. S. P. tests for sterility. Batches were then stored in the dark both at room temperature, 25° ± 4°, and at 45°.

In determining the folic acid and vitamin $B_{12}$ concentrations of the solutions before and after storage, slight changes of published analytical procedures were necessary because of the presence of the additional ingredients. The U. S. P. assay for folic acid was modified by using a fivefold increase in concentration in three of the reagents: sodium nitrite, 1:200; ammonium sulfamate, 1:40; and N-(1-naphthyl)-ethylenediamine dihydrochloride, 1:200. It was found that unless the concentrations of all three reagents were increased as indicated, low results were obtained.

For the determination of vitamin $B_{12}$, three recently developed chemical methods were tested: (*a*) the method of Boxer and Rickards (8), based on the quantitative liberation of the cyano group by photolysis and the subsequent isolation and colorimetric determination of the liberated hydrogen cyanide; (*b*) the method of Rudkin and Taylor (9), which employs the formation of the dicyanide complex of vitamin $B_{12}$, its extraction with benzyl alcohol, and its spectrophotometric differentiation from vitamin $B_{12}$; and (*c*) the method of Marsh and Kuzel (10), founded on the removal of interfering substances by passage through a column of mixed cation-anion exchange resins and subsequent spectrophotometric determination of the vitamin $B_{12}$ in the eluate at a wave-length of 550 mμ. The first two methods are particularly suitable where vitamin $B_{12}$ analogs are present, but they consume much more time than the third method. Since only crystalline vitamin $B_{12}$ was used in the present study, the third method was selected, and proved suitable with slight modifications for determining the concentration of vitamin $B_{12}$ present in the solution. Marsh and Kuzel found the precision of the method to be about 2%, even in the presence of folic acid, the B vitamins, and Vitamin $B_{12}$ decomposition products.

The modified assay procedure consisted of passing the sample solutions through a shortened 50-cc. burette which contained a column of resins consisting of a 10-cm. layer of Amberlite IRA-400 overlaid with a 7-cm. bed of a mixture of equal parts by weight of Amberlite IRA-400 and IR-120. The IRA-400, an anion exchange resin, was previously regenerated with 10% sodium hydroxide solution, and the IR-120, a cation exchange resin, was regenerated with 10% sulfuric acid. Both columns were thoroughly washed with water. In each instance, a sample representing approximately 300 μg. of vitamin $B_{12}$ was passed through the column (to adsorb folic acid and extraneous substances), and the vitamin $B_{12}$ eluate was collected in a 25-cc. volumetric flask which contained 1 cc. of *p*H 5.4 Coleman buffer solution. The flow was regulated to 0.8 cc./min. The column was washed with enough 5% sodium chloride solution to bring the volume up to 25 cc. Spectrophotometric readings were taken in 1-cm. quartz cells at 361 mμ, using a Beckman Model DU spectrophotometer.

By washing with 5% sodium chloride solution, instead of water as originally proposed, the vitamin $B_{12}$ recovered from the resin column was brought up to 95%. The recovery factor is constant for the same set of conditions and may be utilized in computing the results. The wave-length of 361 mμ was found to give a threefold increase in sensitivity over the 550 mμ wave-length suggested.

Comparison was also made with microbiological assays,[1] using the method for determining vitamin $B_{12}$ activity of the U. S. P., Third Supplement.

Data on conditions of the solutions after storage are given in Tables I and II.

## DISCUSSION AND SUMMARY

1. Folic acid can be brought into aqueous solution at a concentration of 5 mg. per cc. and a *p*H of 6.0 by lowering the *p*H of an alkaline folate solution (*p*H 8.5) with dilute hydrochloric acid. Though there is no significant loss of folic acid on storage, the solution tends to turn cloudy after several weeks. The same condition occurs at *p*H 6.5 except that the appearance of the solution after storage is slightly less cloudy.

2. In the presence of 10 per cent glycine or 10 per cent niacinamide, it is possible to bring folic acid into aqueous solution indirectly as described, or directly by heating an aqueous folic acid suspension to 75° with the solubilizer, adjusting to *p*H 6 with dilute sodium hydroxide solution, and cooling to 25°. A concentration of 5 mg. per cc. is achieved. However, after several weeks' storage, the glycine tends to discolor the solution, forming at first a brown colloidal solution, and then a brown gelatinous precipitate. The use of niacinamide permits the solution to remain stable and clear.

3. In the presence of vitamin $B_{12}$ at a concentration of 30 μg. per cc., folic acid solution at a concentration of 5 mg. per cc. remains clear during storage only if certain solubilizing or stabilizing agents are present. At *p*H 6.5 the addition of methyl glucamine, dextrose, or niacinamide in sufficient concentration maintains clarity. At *p*H 6.0, only niacinamide is effective.

4. Parenteral solutions containing vitamin $B_{12}$ and folic acid in the concentrations given do not

[1] Assays were made through the courtesy of Dr. Irving Levenstein of Leberco Laboratories.

TABLE I.—STABILITY OF FOLIC ACID IN INJECTION VITAMIN $B_{12}$ AND FOLIC ACID[a]

| Sample | Solubilizer | pH | Loss of Folic Acid, %: 3 Wk., 45° | Loss of Folic Acid, %: 6 Mo., 25° ± 4 | Clarity of Solutions: 3 Wk., 45° | Clarity of Solutions: 6 Mo., 25° ± 4 |
|---|---|---|---|---|---|---|
| A | None | 6.0 | 1.1 | 2.9 | Cloudy | Cloudy |
| B | None | 6.5 | 1.1 | 2.7 | Cloudy | Opalescent |
| C | Dextrose, 10% | 6.0 | 6.1 | 5.0 | Cloudy | Cloudy |
| D | Dextrose, 10% | 6.5 | 7.8 | 4.6 | Clear | Clear |
| E | Methyl glucamine, 1% | 6.0 | 6.0 | 5.1 | Ppt. | Cloudy |
| F | Methyl glucamine, 1% | 6.5 | 5.1 | 4.5 | Clear | Clear |
| G | Glycine, 10% | 6.0 | 3.7 | 3.0 | Gel. ppt. | Gel. ppt. |
| H | Glycine 10% | 6.5 | 4.2 | 2.7 | Gel. ppt. | Gel. ppt. |
| I | Niacinamide, 10% | 6.0 | 5.9 | 3.9 | Clear | Clear |
| J | Niacinamide, 10% | 6.5 | 1.0 | 2.4 | Clear | Clear |
| K[b] | None | 6.0 | 5.5 | 3.0 | Cloudy | Gloudy |
| L[b] | None | 6.5 | 1.5 | 2.4 | Opalescent | Opalescent |

[a] 5 mg. of folic acid and 30 μg. of vitamin $B_{12}$ per cc.
[b] Controls containing 5 mg. per cc. of folic acid but no vitamin $B_{12}$.

TABLE II.—STABILITY OF VITAMIN $B_{12}$ IN INJECTION VITAMIN $B_{12}$ AND FOLIC ACID[a]

| Sample | Solubilizer | pH | Loss of Vitamin $B_{12}$ (Chem.), %: 3 Wks., 45° | Loss of Vitamin $B_{12}$ (Chem.), %: 6 Mo., 25° ± 4 | Loss of Vitamin $B_{12}$ (Microbiol.), %: 3 Wks., 45° | Loss of Vitamin $B_{12}$ (Microbiol.), %: 6 Mo., 25° ± 4 |
|---|---|---|---|---|---|---|
| A | None | 6.0 | 0 | 2.1 | 2 | 0 |
| B | None | 6.5 | 0 | 2.2 | 3 | 2 |
| C | Dextrose, 10% | 6.0 | 2.0 | 3.4 | 5 | 4 |
| D | Dextrose, 10% | 6.5 | 12.3 | 10.0 | 14 | 9 |
| E | Methyl glucamine, 1% | 6.0 | 6.0 | 5.0 | 7 | 7 |
| F | Methyl glucamine, 1% | 6.5 | 12.0 | 10.2 | 13 | 12 |
| G | Glycine, 10% | 6.0 | 4.7 | 3.6 | 7 | 5 |
| H | Glycine, 10% | 6.5 | 9.0 | 7.4 | 10 | 8 |
| I | Niacinamide, 10% | 6.0 | 0 | 0 | 2 | 2 |
| J | Niacinamide, 10% | 6.5 | 0 | 2.1 | 3 | 2 |
| M[b] | None | 6.0 | 3.3 | 2.4 | 2 | 2 |
| N[b] | None | 6.5 | 4.0 | 3.2 | 2 | 2 |

[a] 5 mg. of folic acid and 30 μg. of vitamin $B_{12}$ per cc.
[b] Controls containing 30 μg. of vitamin $B_{12}$ per cc. but no folic acid. These samples remained clear during storage at room temperature and at 45°.

show material losses of folic acid during storage for three weeks at 45° or six months at room temperature and *p*H 6 or *p*H 6.5, either in the presence or the absence of the solubilizers studied. The greatest loss was only 7.8 per cent in the presence of dextrose, at *p*H 6.5.

5. Under similar conditions, there was no loss of vitamin $B_{12}$ due to folic acid alone. With respect to the effect of solubilizing and stabilizing agents, the losses ranged from about 12 per cent in the presence of methyl glucamine and dextrose to a minimum of about 2 per cent when niacinamide was the solubilizer. In this connection it is interesting to point out that Macek and Feller (11), in studying the compatibility of vitamin $B_{12}$ with other therapeutic agents, found that after a year's storage at room temperature, a loss of only 3 per cent of vitamin $B_{12}$ occurred in the presence of niacinamide, using concentrations of 5–20 μg. of vitamin $B_{12}$ per cc. at *p*H 4. No folic acid combinations were included in the study.

6. It may be concluded that parenteral solutions can be prepared at concentrations of 30 μg. of vitamin $B_{12}$ and 5 mg. of folic acid per cc. at a *p*H of 6 and up to a *p*H of 6.5 in the presence of 10 per cent niacinamide solution as solubilizer. Such solutions may be expected to have a practical shelf life in regard to clarity of the solution and potency of the folic acid and the vitamin $B_{12}$.

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# Effect of Certain Surface Active Agents upon Bacterial Growth*

By LOUIS GERSHENFELD and GILBERT C. JOHNSON

**The character of the growth of rough strains of *Staphylococcus aureus, Streptococcus viridans,* and *Neisseria catarrhalis* was not changed when these organisms were cultured in the presence of 0.001 to 0.0001 per cent of sodium lauryl sulfate. Lecithin in concentrations of 0.003 to 0.01 per cent stimulated the growth of the two Gram-positive organisms but had no effect upon the growth of the Gram-negative organism. Tween 80 in concentrations of 0.3 to 2 per cent caused the rough *Staphylococcus aureus* to grow as a smooth suspension in broth medium but did not change the colony structure on agar medium. The *Streptococcus* and *Neisseria* species were not affected.**

THE macroscopic growth characteristics of a bacterium in broth culture is principally a function of the bacterial surface (8, 11, 19, 20). Many nonmotile bacteria normally produce a homogenous suspension in liquid culture media at the end of a satisfactory growth period. In this respect they possess some characteristics similar to hydrophilic colloid mixtures. Other organisms normally produce a granular type of growth in a liquid culture medium, the individual organisms agglomerating to form macroscopic aggregates which may fall to the bottom of the culture, cling to the side of the container, or form a film at the broth-air interface. This behavior in certain organisms is similar to that of hydrophobic colloidal mixtures (8, 12).

It has been observed that the "homogenous-growing organisms" will, on occasion, produce "granular-growing organisms" (8). This action in part has been compared with a change of state from hydrophilic to hydrophobic, which, in the presence of sufficient electrolyte, might account to some extent for the agglomeration (8–13). In the case of specific organisms, it has been shown that hydrophobic substances are exposed in these changes (13, 14).

In studies made on granular-growing and pellicle-producing bacteria, several investigators have used surface tension depressants. Dubos (4–6) obtained homogenous growth in several species of *Mycobacterium*, including *M. tuberculosis*, by the addition of surface tension depressants. Ward (15) found that Tween 80 caused *Corynebacterium diphtheriae* var. *minimus* to grow as a homogenous suspension. Larson, *et al.* (9, 10), reported that the use of several surface tension depressants resulted in the growth of *Bacillus subtilis* in the body of a broth culture rather than as a surface pellicle.

Mellon, *et al.* (19), made detailed studies on the effect of interfacial tension reduction upon emulsification of a diphtheria-like culture, ND-95 which formed large floccules when placed in distilled water or 0.85-per cent sodium chloride solution. With 0.02 *M* sodium oleate in distilled water, this organism formed a smooth homogenous suspension. These results were explained on the basis of strong adsorption of the oleate portion of the molecule on the lipoid-like surface of the bacterium. It was also concluded that the air-liquid interface tension had little effect on the bacterial surface-liquid interfacial tension.

Frobisher (20), using smooth strains of *Staphylococcus* isolated from skin and growing in broth, found that as the surface tension was made lower by increasing the concentration of sodium oleate, clumping of the bacteria increased. The mechanism of the reaction was not discussed.

Baker (1–3) and Hotchkiss (7) demonstrated that the nonionic surface tension depressants either stimulated or did not affect the metabolism of Gram-positive and Gram-negative bacteria.

Little work has been reported in the literature on the possible temporary change of the rough-growing phase of cocci to the smooth-growing phase by use of a surface tension depressant. The present study was made to obtain information on this problem.

## EXPERIMENTAL

The glassware used in the experiments was washed with 5% detergent (Surf[1]), rinsed three times with tap water, cleaned with dichromate cleaning solution (16), and dried and sterilized in a hot air oven at 160° for two hours.

The culture medium used was enzymatic casein digest-yeast extract broth, *p*H 7.4 after sterilization. A solid culture medium was prepared by the addition of 1.5% Bacto-agar to the broth. This medium will be referred to as "the broth" when liquid, and "the agar" when solid.

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[1] Manufactured by Lever Brothers, New York, N. Y.

The organisms used were *Streptococcus viridans* b., *Neisseria catarrhalis*[2] (a rough strain), and *Micrococcus pyogenes* var. *aureus* [a rough variant of 209 (17)]. In this study, these organisms are referred to as *Streptococcus*, *Neisseria*, and *Staphylococcus*.

All of the organisms produced macroscopically visible growth in the culture medium at the end of twenty-four hours' incubation at 37°. The organisms were maintained as stock cultures on agar slants. These were transplanted each week to fresh slants of agar, incubated for twenty-four hours at 37°, and stored at 6–8°. Transplants were made in 20 ml. of the broth twenty-four hours previous to use and one standard 4-mm. loopful (17) of this broth culture was used to seed all broth tubes. The agar surface was inoculated with a loopful from a 1:100 dilution of the broth culture (diluted with the broth). All broth cultures were examined macroscopically to confirm granular growth before use.

The surface tension depressants selected for this study were sodium lauryl sulfate, m. p. 189, SH-259[3]; Tween 80, Lot 110 A[4]; and lecithin from soy bean. These agents have been shown to stimulate metabolic activity of bacteria (1, 3).

Tween 80 and sodium lauryl sulfate solutions were prepared as 10% and 1% solutions in the broth. Lecithin was prepared in ethyl ether U. S. P. in 10% and 1% solutions.

The prepared solutions were passed through a Pyrex fritted-disk filter (UF-36060[5]), stored in sterile screw cap vials at 6–8°, and tested for sterility by running uninoculated controls with each test.

fluid (50°) into sterile Petri dishes. Both broth and agar controls were prepared and consisted of the media without the surface active agents.

The test tubes and Petri dishes containing the sterile surface active agent-culture medium mixtures were inoculated, using the standard 4-mm. loop (17), and incubated for twenty-four hours at 37°. Negative controls containing no organisms but including the surface active agents were prepared. Before inoculation the dilutions and controls were incubated twenty-four hours at 37° to note sterility.

The diameters of the colonies were measured with an ocular micrometer and a compound microscope. The micrometer was standardized with a blood-cell counting chamber so that 35 micrometer scale markings were equivalent to 3 mm., or one scale marking was equivalent to 0.086 mm. Colonies were measured only if they were at least 5 mm. distant from the nearest colony. Five colonies were measured on each plate.

## PROCEDURE

**Preliminary Experiment.**—Before any observation could be made of the effect on the bacteria of the agents under test, it was necessary to determine what concentrations of the agents would be satisfactory. Dilutions of the surface active agents were prepared in the broth as indicated in Table I, Parts A, B, and C.

All cultures were incubated for seventy-two hours at 37° and examined macroscopically for growth.

TABLE I.—GROWTH RESULTS IN BROTH CONTAINING VARIOUS DILUTIONS OF SURFACE ACTIVE AGENTS AND INOCULATED WITH BACTERIA[a,b]

A

| Organism | Tween 80 in Broth, % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2.0 | 1.7 | 1.5 | 1.3 | 0.7 | 0.5 | 0.3 | 0.1 | 0 |
| *Staphylococcus* | G[c] | G | G | G | G | G | G | G | G |
| *Streptococcus* | G | G | G | G | G | G | G | G | G |
| *Neisseria* | G | G | G | G | G | G | G | G | G |

B

| Organism | Sodium Lauryl Sulfate in Broth, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1.0 | 0.5 | 0.1 | 0.05 | 0.01 | 0.005 | 0.001 | 0 |
| *Staphylococcus* | 0[d] | 0 | 0 | 0 | 0 | 0 | G | G |
| *Streptococcus* | 0 | 0 | 0 | 0 | 0 | 0 | G | G |
| *Neisseria* | 0 | 0 | 0 | 0 | 0 | 0 | G | G |

C

| Organism | Lecithin in Broth, % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2.0 | 1.7 | 1.5 | 1.3 | 1.0 | 0.1 | 0.01 | 0.001 | 0 |
| *Staphylococcus* | T[e] | T | T | T | T | T | G | G | G |
| *Streptococcus* | T | T | T | T | T | T | G | G | G |
| *Neisseria* | T | T | T | T | T | T | G | G | G |

[a] All cultures were incubated 24 hours at 37° and examined macroscopically for growth.
[b] All per cent figures indicate w/v concentrations of the surface active agent in enzymatic casein digest-yeast extract broth, pH 7.4.
[c] G indicates growth.
[d] 0 indicates no growth.
[e] T indicates that the medium was turbid before inoculation and so was not inoculated.

**Technique.**—Dilutions of the surface active agents were made in test tubes containing the broth. Fifteen-milliliter volumes of the agar were treated in the same way as the broth and poured while still

[2] Strains isolated from throat cultures of students at the Philadelphia College of Pharmacy and Science.
[3] E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.
[4] Atlas Powder Co.
[5] Corning Glass Co.

**Findings.** The *Staphylococcus*, *Streptococcus*, and the *Neisseria* produced growth in concentrations of the Tween 80 ranging from 0.1 to 2%.

With sodium lauryl sulfate, the *Staphylococcus* and the *Streptococcus* produced growth in concentrations of only 0.001 per cent or less. The *Neisseria* produced growth in concentrations of 0.005% or less.

The concentrations of lecithin above 0.01% were turbid before inoculation, due to the poor solubility

of the lecithin in the broth. Only those concentrations giving a clear solution were inoculated. All three organisms produced growth in concentrations of 0.01 and 0.001%.

Accordingly the following ranges of dilutions of the surface active agents in the broth were used in this study: Tween 80, 0.1 to 2%; lecithin, 0.01 to 0.001%; and sodium lauryl sulfate, 0.001 to 0.0001%.

**Experiment I: Effects of Sodium Lauryl Sulfate upon the Growth of Various Bacteria in Liquid Culture Medium.**—Four series of dilutions were prepared as shown in Table II. Series 1 was inoculated with the *Staphylococcus;* Series 2 with the *Streptococcus;* and Series 3 with the *Neisseria.* Series 4 was not inoculated. The cultures used for the inoculation were prepared as previously outlined. With a sterile pipette, 0.05 ml. of the culture was added to the tubes containing 5 ml. of the broth-surface active agent mixture. All tubes were incubated for twenty-four hours and examined macroscopically for growth characteristics.

**Findings: Experiment I.**—There was no marked difference between the character of the growth resulting in the tubes containing the diluted sodium lauryl sulfate and the controls.

The series inoculated with *Streptococcus* and the one inoculated with *Staphylococcus* showed less growth with increasing concentration in the range explored.

The series inoculated with *Neisseria* showed no apparent difference in quantity of growth in the range examined.

**Experiment II: The Effects of Sodium Lauryl Sulfate upon the Growth of Various Bacteria on Solid Culture Medium.**—Four series of dilutions were made as shown in Table III. These dilutions were prepared in melted agar at 50° and poured while still liquid into Petri dishes. After solidification, the surface of the agar was inoculated as outlined under Procedure C, with the same organisms used in Experiment I. One series was not inoculated. All cultures were incubated for twenty-four hours at 37°, and then were examined for growth characteristics.

**Findings: Experiment II.**—There was no marked difference between the colonies produced on the agar containing the surface active agents and those produced on the agar without the agent, with the exception of colony size.

As the concentration of the agent was increased, the average colony diameter became less with the *Streptococcus* and the *Staphylococcus.*

The *Neisseria* colony size remained about the same, as indicated in Table III.

**Experiment III: The Effects of Tween 80 upon the Growth of Various Bacteria in Liquid Culture Medium.**—Four series of dilutions were prepared as shown in Table I, Part A. They were inoculated and incubated as in Experiment I.

**Findings: Experiment III.**—There was no change in the character of the growth of the *Streptococcus* and the *Neisseria* resulting in the Tween 80 dilutions and the control tubes.

The growth of the *Staphylococcus* appeared to be progressively less granular as the concentration of the Tween 80 was increased, starting with the 0.3% tube. The concentrations between 0.7% and 2% appeared similar in character with a homogenous suspension of the bacteria in the supernatant broth and a fine powdery sediment of the bacteria on the bottom. The growth in the 0.1% concentration was identical with that in the control tube.

**Experiment IV.**—Transplants were made from all of the *Staphylococcus* cultures obtained in Experiment III to the tubes of broth containing no surface active agents. These cultures were incubated for twenty-four hours at 37°, and were then examined for macroscopic growth characteristics.

**Findings: Experiment IV.**—In each case, the growth resulting was granular and usually sedimented, with a slight pellicle on the surface of the medium.

**Experiment V: The Effects of Tween 80 upon the Growth of Various Bacteria on Solid Culture Medium.**—Four series of dilutions were prepared as indicated in Table III, Part B. They were inoculated and incubated as in Experiment II.

**Findings: Experiment V.**—There was no apparent difference in the isolated colonies produced on the agar containing the Tween 80 dilutions and the controls. Measurements of the diameters of the colonies indicated that there were no unusual variations in size.

TABLE II.—CHARACTERISTICS OF GROWTH IN BROTH CONTAINING VARIOUS DILUTIONS OF SODIUM LAURYL SULFATE AND INOCULATED WITH VARIOUS BACTERIA[a,b]

| Organism | Sodium Lauryl Sulfate in Broth, % | | | | | |
|---|---|---|---|---|---|---|
| | 0.001 | 0.0007 | 0.0005 | 0.0003 | 0.0001 | 0 |
| *Staphylococcus* | G[c] | G | G | G | G | G |
| | P[d] | P | P | P | P | P |
| | S[e] | S | S | S | S | S |
| *Streptococcus* | F[f] | F | F | F | F | F |
| | S | S | S | S | S | S |
| *Neisseria* | C[g] | C | C | C | C | C |
| | P | P | P | P | P | P |
| | S | S | S | S | S | S |
| Not inoculated | 0[h] | 0 | 0 | 0 | 0 | 0 |

[a] All cultures incubated 24 hours at 37° and examined macroscopically for growth.
[b] All per cent figures indicate w/v concentrations in enzymatic casein digest-yeast extract broth, *p*H 7.4.
[c] G indicates fine granular growth on bottom and sides of tube.
[d] P indicates pellicle at surface.
[e] S indicates clear supernatant broth.
[f] F indicates flocculent growth on bottom of tube.
[g] C indicates coarse granular growth on bottom and sides of tube.
[h] 0 indicates no growth.

**Experiment VI: The Effects of Lecithin upon the Growth of Various Bacteria in Liquid Culture Medium.**—Four series of dilutions were prepared as in Table IV. They were inoculated and incubated as in Experiment II.

**Findings: Experiment VI.**—There was no marked difference in the character of the growth between the tubes containing the lecithin dilutions and the controls. However, the amount of growth varied.

The series inoculated with *Streptococcus* and *Staphylococcus* appeared to produce a heavier growth than the control tubes, while the series inoculated with *Neisseria* did not differ from the controls.

**Experiment VII: The Effect of Lecithin upon the Growth of Various Bacteria on Solid Culture Medium.**—Four series of dilutions were prepared as shown in Table III, Part C. They were inoculated and incubated as in Experiment II.

**Findings: Experiment VII.**—There was no difference between the colonies of *Neisseria* on the lecithin-containing plates and the control plates, as indicated in Table III, Part C.

TABLE III.—MEAN COLONY DIAMETERS IN MM. OF DIFFERENT BACTERIA GROWING ON AGAR MEDIUM CONTAINING DILUTIONS OF SURFACE ACTIVE AGENTS[a,b]

Part A

| | Concn. Sodium Lauryl Sulfate in Broth, % | | | |
|---|---|---|---|---|
| Organism | 0.001 | 0.0005 | 0.0001 | 0 |
| *Staphylococcus* | 1.7 | 1.9 | 2.2 | 2.3 |
| t score | 2.44 | 2.19 | 0.53 | ... |
| *Streptococcus* | 0.4 | 0.5 | 0.7 | 0.8 |
| t score | 7.35 | 4.17 | 0.50 | ... |
| *Neisseria* | 1.3 | 1.7 | 1.5 | 1.5 |
| t score | 1.64 | 1.19 | ... | ... |

Part B

| | Concn. Tween 80 in Broth, % | | | | |
|---|---|---|---|---|---|
| Organism | 1.5 | 1.0 | 0.5 | 0.1 | 0 |
| *Staphylococcus* | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 |
| t score | ... | ... | ... | ... | ... |
| *Streptococcus* | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| t score | ... | ... | ... | ... | ... |
| *Neisseria* | 1.6 | 1.5 | 1.4 | 1.7 | 1.5 |
| t score | 1.45 | ... | 1.39 | 1.57 | ... |

Part C

| | Concn. Lecithin in Broth, % | | | | |
|---|---|---|---|---|---|
| Organism | 0.01 | 0.007 | 0.003 | 0.001 | 0 |
| *Staphylococcus* | 3.1 | 3.0 | 2.5 | 2.4 | 2.4 |
| t score | 2.22 | 1.79 | 0.61 | ... | ... |
| *Streptococcus* | 0.9 | 0.9 | 0.8 | 0.7 | 0.7 |
| t score | 1.84 | 1.78 | 0.6 | ... | ... |
| *Neisseria* | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| t score | ... | ... | ... | ... | ... |

[a] All means are averages of five colonies, each not closer than 5 mm. to the next nearest colony.
[b] 5% value of P = 2.78.

TABLE IV.—CHARACTERISTICS OF GROWTH IN BROTH CONTAINING VARIOUS DILUTIONS OF LECITHIN AND INOCULATED WITH VARIOUS BACTERIA[a,b,c]

| | Concn. of Lecithin in Broth, % | | | | |
|---|---|---|---|---|---|
| Organism | 0.01 | 0.007 | 0.003 | 0.001 | 0 |
| *Staphylococcus* | G[d] | G | G | G | G |
| | P[e] | P | P | P | P |
| | S[f] | S | S | S | S |
| | 1.92 | 1.76 | 1.46 | 1.0 | 1.0 |
| *Streptococcus* | F[g] | F | F | F | F |
| | S | S | S | S | S |
| | 1.98 | 1.88 | 1.34 | 1.0 | 1.0 |
| *Neisseria* | C[h] | C | C | C | C |
| | P | P | P | P | P |
| | S | S | S | S | S |
| Not inoculated | 0[i] | 0 | 0 | 0 | 0 |

[a] All cultures were incubated for 24 hours at 37° and examined macroscopically for growth.
[b] The per cent figures indicate w/v of lecithin in enzymatic casein digest-yeast extract broth.
[c] The numbers in the body of the chart indicate the extent to which the culture was diluted with homologous broth so that the bacterial density appeared equal to that of the control. However this could not be done with the *Neisseria* due to the character of the growth.
[d] G indicates fine granular growth on the bottom and sides of the tube.
[e] P indicates pellicle at surface.
[f] S indicates clear supernatant broth.
[g] F indicates flocculent growth on the bottom of the tube.
[h] C indicates coarse granular growth on bottom and side of the tube.
[i] 0 indicates no growth.

TABLE V.—CHARACTERISTICS OF GROWTH IN BROTH CONTAINING VARIOUS DILUTIONS OF TWEEN 80 AND INOCULATED WITH DIFFERENT BACTERIA[a,b]

| Organism | Concn. of Tween 80 Broth, % 2.0 | 1.7 | 1.5 | 1.3 | 0.7 | 0.5 | 0.3 | 0.1 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| *Staphylococcus* | H[c] | H | H | H | H | SG[d] | SG | G[e] | G |
| | R[f] | R | R | R | R | R | P[g] | P | P |
| *Streptococcus* | F[h] | F | F | F | F | F | F | F | F |
| | S[i] | S | S | S | S | S | S | S | S |
| *Neisseria* | C[j] | C | C | C | C | C | C | C | C |
| | P | P | P | P | P | P | P | P | P |
| | S | S | S | S | S | S | S | S | S |
| Not inoculated | 0[k] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] All cultures were incubated for 24 hours at 37° and examined macroscopically for growth.
[b] The per cent figures indicate w/v of Tween 80 in enzymatic casein digest-yeast extract broth.
[c] H indicates homogenous growth.
[d] SG indicates a slightly granular growth.
[e] G indicates a fine granular growth on bottom and sides of tube.
[f] R indicates fine powdery sediment on bottom of tube.
[g] P indicates pellicle at surface.
[h] F indicates flocculent growth on bottom of tube.
[i] S indicates clear supernatant broth.
[j] C indicates coarse granular growth on bottom and sides of tube.
[k] 0 indicates no growth.

The colonies produced by the *Staphylococcus* and the *Streptococcus* were larger with increasing concentrations of lecithin, as indicated in Table III, Part C.

## DISCUSSION

Several authors have used surface active agents to obtain smooth growth of pellicle-forming and rough-growing bacteria (4, 9, 15). Other than *Bacillus subtilis*, the organisms used contained a high concentration of lipid material of hydrophobic nature. Adsorption of the surface active agent at the bacterial-liquid interface with a resulting change of character to hydrophilic has been advanced as the mechanism of the reaction (19). Many rough variants have lost their hydrophilic surface and exist in the rough phase because of the increase in lipid groupings exposed at their new surface (12–14, 18). The granular consistency of many of the rough types is accounted for, in part, by post-fission movements of the cells (18).

The *Staphylococcus* used in these experiments evidently falls into the group of rough organisms which find the increased lipids in the exposed surface partly responsible for the hydrophobic nature of the cells. Why the lecithin and sodium lauryl sulfate did not duplicate the effect of the Tween 80 is not known.

In some respects, the work of Frobisher (20) contradicts these results, but as he was working with a colloidal electrolyte (sodium oleate) and smooth-growing bacteria, several physical-physiological factors may have been responsible for the clumping of the cells.

Because the *Streptococcus* was not affected by the Tween 80, it evidently falls into the group of organisms whose rough growth is due, to some extent, to the morphology (interlacing chains in this case).

Why the *Neisseria* grows rough is not known.

## SUMMARY AND CONCLUSIONS

1. Rough strains of *Staphylococcus aureus*, *Streptococcus viridans*, and *Neisseria catarrhalis* were cultured separately in broth and on agar medium in the presence of surface active agents.

2. The character of the growth of the three rough-growing organisms examined was not changed when these organisms were cultured in the presence of 0.001 to 0.0001 per cent sodium lauryl sulfate. Higher concentrations were unsatisfactory because of the toxicity of the compound for the bacteria used.

3. Lecithin in concentrations of 0.003 to 0.01 per cent stimulated the growth of the two Gram-positive organisms but had no effect upon the growth of the Gram-negative organism. Higher concentrations of lecithin were not used because the 0.01 per cent represented the upper limit of solubility in the culture medium.

4. Tween 80 in concentrations of 0.3 to 2 per cent caused the rough *Staphylococcus* to grow as a smooth suspension in the broth, but did not change the colony structure on agar. In the same concentration, there was no effect upon the *Streptococcus* and the *Neisseria*.

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# The Solubility of Sucrose in Hydroalcoholic Solutions*

By LOUIS A. REBER

**A report is presented on the solubility of sucrose in hydroalcoholic solutions, measured at 25° over the entire range of alcohol concentrations at intervals of approximately 10 per cent. Information is included concerning the effect of alcohol on the specific rotation of sucrose.**

INFORMATION on the solubility of sucrose in hydroalcoholic solutions is incomplete. Seidell (1) reports data at 0, 14, and 40°, based on work done in the last century. Data at room temperature are entirely lacking. The International Critical Tables (2) contain similar information and also give the solubility of sucrose in 80 per cent alcohol (by volume) at 20° as 37 Gm./L. of solution. This figure is based on the work of Hudson and Yanovsky (3), who claim that saturation was attained within five minutes after mixing. The solubility reported by these two workers appears to be entirely too low when compared with the values at other temperatures.

The present investigation was undertaken to supply data on the solubility of sucrose in hydroalcoholic solutions at 25°. The entire range of alcohol concentrations, at intervals of approximately 10 per cent, has been covered (Table I).

## EXPERIMENTAL

**Materials.**—Hydroalcoholic solutions were prepared from distilled water and alcohol U. S. P. Absolute alcohol was also used as a solvent. In all cases, the percentage of alcohol was determined from measurement of the specific gravity, using a 50-ml. pycnometer.

Sucrose was obtained from the National Bureau of Standards. It was checked for optical rotation and was used in the solubility determinations without further purification.

**Method.**—Determinations of solubility were performed by agitating an excess of solute with solvent contained in glass-stoppered bottles sealed with paraffin, in a bath maintained at 25° ± 0.01°. The period of agitation was forty-eight hours or longer. The amount of sucrose dissolved was determined by evaporating a weighed amount of saturated solution to dryness and drying the residue in a vacuum oven at 60°. The drying was continued until the residues reached essentially constant weight.

At the same time that a sample of saturated solution was removed for analysis, another portion was used to determine the specific gravity in order to permit calculation of solubilities on both weight and volume bases.

Measurements of the optical rotation of sucrose solutions were made to determine the effect of alcohol on the specific rotation of sucrose. As much as 50% (by volume) of alcohol had no demonstrable effect; higher percentages caused an increase in specific rotation. In 80% alcohol (by volume) the specific rotation, $[\alpha]_D^{25}$, rose to 67.5 as compared to 66.5 in aqueous solution. Browne and Zerban (4) report a similar but less pronounced effect of alcohol on the specific rotation of sucrose.

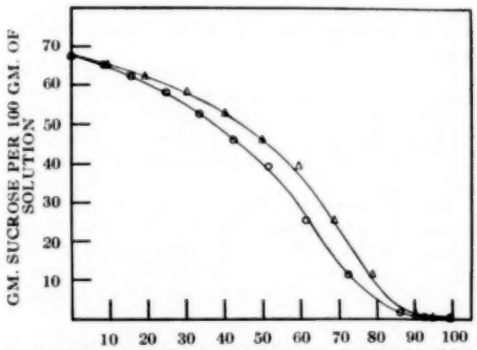


Fig. 1.—The solubility of sucrose expressed as Gm. of sucrose in 100 Gm. of solution.

* Received September 5, 1952, from the Chemistry Department, Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

TABLE I.—THE SOLUBILITY OF SUCROSE IN HYDROALCOHOLIC SOLUTIONS

| Alcohol in Solvent, Weight % | Alcohol in Solvent, Volume %[a] | Solubility as Gm. of Sucrose in 100 Gm. of Soln. | Solubility as Gm. of Sucrose in 100 Ml. of Soln. | Solubility as Gm. of Sucrose in 100 Gm. of Solvent | Solubility as Gm. of Sucrose in 100 Ml. of Solvent | Density of Satd. Soln., Gm./ml. |
|---|---|---|---|---|---|---|
| 0.00 | 0.00 | 67.6 | 89.7 | 208.6 | 207.7 | 1.3274 |
| 8.62 | 9.48 | 65.2 | 85.1 | 187.4 | 184.2 | 1.3054 |
| 15.79 | 19.42 | 62.2 | 79.5 | 164.8 | 160.0 | 1.2775 |
| 24.88 | 30.24 | 58.1 | 72.0 | 138.4 | 132.6 | 1.2411 |
| 33.53 | 40.18 | 52.9 | 63.3 | 112.2 | 105.9 | 1.1976 |
| 42.48 | 49.99 | 46.1 | 52.8 | 85.7 | 79.3 | 1.1443 |
| 51.83 | 59.67 | 39.6 | 43.4 | 65.6 | 59.4 | 1.0949 |
| 61.30 | 68.93 | 25.4 | 25.3 | 33.3 | 30.0 | 0.9948 |
| 72.38 | 79.00 | 11.39 | 10.32 | 12.85 | 11.01 | 0.9060 |
| 86.15 | 90.36 | 1.675 | 1.391 | 1.703 | 1.401 | 0.8307 |
| 92.50 | 95.05 | 0.401 | 0.324 | 0.403 | 0.325 | 0.8070 |
| 99.42 | 99.65 | 0.065 | 0.051 | 0.065 | 0.051 | 0.7865 |

[a] At 15.56°.

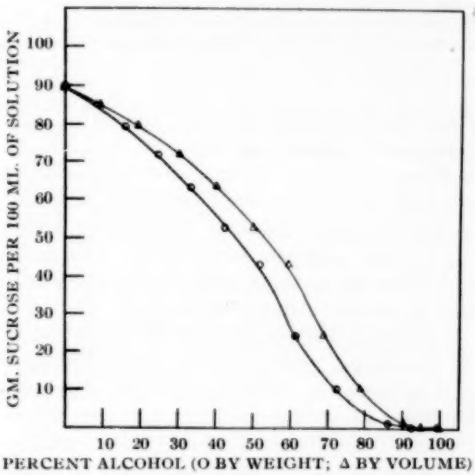
GM. SUCROSE PER 100 ML. OF SOLUTION
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70 80 90 100
PERCENT ALCOHOL (O BY WEIGHT; Δ BY VOLUME)

Fig. 2.—The solubility of sucrose expressed as Gm. of sucrose in 100 ml. of solution.

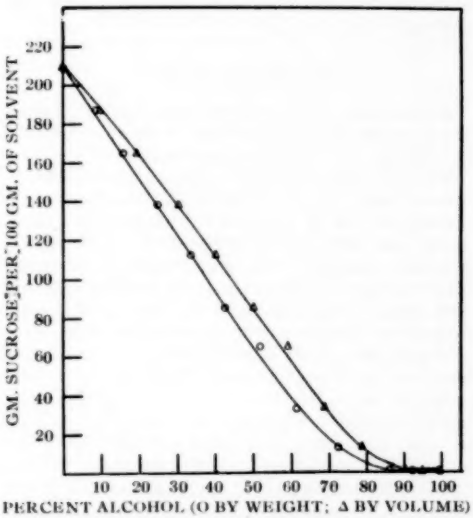
GM. SUCROSE PER 100 GM. OF SOLVENT
20 40 60 80 100 120 140 160 180 200 220
10 20 30 40 50 60 70 80 90 100
PERCENT ALCOHOL (O BY WEIGHT; Δ BY VOLUME)

Fig. 3.—The solubility of sucrose expressed as Gm. of sucrose in 100 Gm. of solvent.

## RESULTS

The results of the solubility measurements are given in Table I, and are expressed graphically in Figs. 1–4; all measurements are at 25° unless otherwise noted.

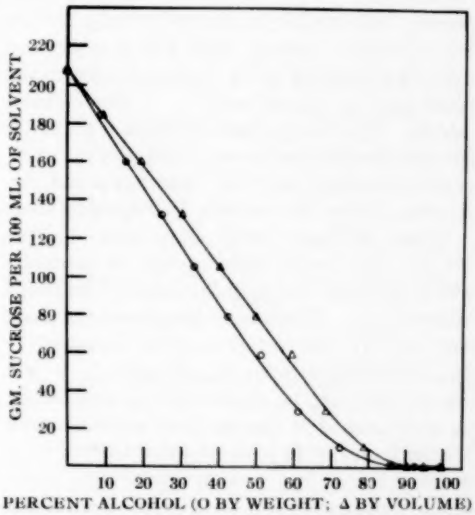
GM. SUCROSE PER 100 ML. OF SOLVENT
20 40 60 80 100 120 140 160 180 200 220
10 20 30 40 50 60 70 80 90 100
PERCENT ALCOHOL (O BY WEIGHT; Δ BY VOLUME)

Fig. 4.—The solubility of sucrose expressed as Gm. of sucrose in 100 ml. of solvent.

## SUMMARY

The solubility of sucrose in hydroalcoholic solutions has been determined at 25°. The entire range of alcohol concentrations, at intervals of approximately 10 per cent, has been covered.

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# The Physics of Tablet Compression. II. The Influence of Degree of Compression on Properties of Tablets*†‡

By T. HIGUCHI, A. NARSIMHA RAO,§ L. W. BUSSE, and J. V. SWINTOSKY

**A study has been made of the influence of compressional force employed in tablet compression on the apparent density, porosity, hardness, disintegration time, and average primary particle size of compressed tablets. The data obtained for the sulfathiazole granulations studied indicate that there is a logarithmic relationship between the compressional force and the apparent density or the porosity of the tablets and that the pressure distribution inside the die cavity during the compressional process appears to be quite uniform. Neither hardness nor disintegration time seems to be strongly influenced by granulation size distribution. The following appear to have linear relationships: the plot between Strong Cobb Hardness and the logarithm of the compressional force; hardness and porosity; and the logarithm of disintegration time when plotted against the compressional force. The specific surface areas of compressed tablets undergo marked changes during the compressional process, exhibiting pronounced maxima at 2,500 lbs. for ³/₈-inch tablets.**

In the introductory paper (1) of this series it was stated that the factors affecting compressed tablets may be advantageously divided into two types, namely, static and dynamic, and must be correlated in the final analysis with the disintegrating characteristics of the formed tablets. The present communication is primarily concerned with experimental findings of the static type as obtained on a fixed formulation and correlation of these factors with disintegrating time.

Many previous studies have been directed toward the general investigation of variables which influence the manufacture of compressed tablets (2–6). Others have been more concerned with such specific factors as tablet hardness (7–16), tablet disintegration time (6, 9, 13, 17–19), and age of tablets (6, 13, 18, 20–22). In spite of the obvious interest in the practical aspects of this problem, very little attempt seems to have been made as late as 1950 toward quantitative correlation of more than two variables of manufacture or properties.

In the present investigation, an attempt has been made to establish the relationship between the maximal force employed during the compressional process and several tablet properties including (*a*) apparent density and porosity; (*b*) particle size; (*c*) hardness; and (*d*) disintegration time. From the manufacturing and distributing standpoint, primary concern must be given to hardness, which is a measure of resistance to chipping, abrasion, or breakage under the conditions of storage, transport, and handling before usage, and to disintegration time. The other properties are more concerned with the fundamental process of compression.

No attempt has been made in the present study to formulate a general theory on the formation and properties of compressed tablets, since such an undertaking requires more fundamental information, both theoretical and experimental, on the behavior of granular matter under compressive force. The purpose of this report is to present basic experimental evidence so that semi-empirical relationships of value to workers in the field may be developed.

## EXPERIMENTAL

**Preparation of Tablets.**—A single type of sulfathiazole formulation was used to prepare granules and compressed tablets of sulfathiazole.

A starch paste (10%) was prepared by boiling 20 Gm. of cornstarch in 200 cc. of distilled water to the consistency of a relatively homogeneous paste, which was added to sulfathiazole U. S. P. in proportions necessary for granulation. The moist mass was granulated by passing it through a No. 10 wire sieve. The granules were dried in a hot-air oven at 60° for four hours, and were then reduced in size with the aid of a Stokes grinder. The product obtained was sieved through a No. 20 screen and collected on a No. 60 screen. The granules collected on the No. 60 screen were relatively uniform in size and assayed 96.9% sulfathiazole. This granulation was stored over anhydrous calcium chloride for at least three days, after which 1% of its weight of cornstarch powder, previously dried at 60° for four hours, was added as a disintegrating agent. A high measure of homogeneity was ob-

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‡ This paper is based in part on a thesis submitted to the Graduate School of the University of Wisconsin by A. N. Rao in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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tained by carrying out this operation on 30-Gm. lots of the granules and then combining and thoroughly mixing the separate lots. The product, designated Batch 1, was stored in a desiccator over calcium chloride for at least three days before compression into tablets. A second batch of granules, designated Batch 2, was prepared and treated in a manner identical to Batch 1 except that they were collected on a No. 40 screen and thus possessed a coarser texture.

To facilitate formation of tablets of uniform size, the granules were then weighed into exactly 0.330-Gm. quantities, equivalent to 5.20 gr. of granules and 5.00 gr. of sulfathiazole. The compression of the granules to tablets was accomplished by the use of a flat head, $^3/_8$-in. die and punch set employed in conjunction with a mechanical lever machine. This equipment, which has been previously described (1), permitted compression of the tablets at exact force levels ranging from several hundred pounds per tablet to levels far in excess of those used in actual tablet manufacture. About 60 tablets were prepared at each compressional force level. The rate of compression with the instrument used produced a cylindrical, flat-surface tablet in about twenty to sixty seconds, depending upon the force level. The force levels used in this study ranged from 500 to 8,000 lb. per tablet.

**Specific Surface Area Measurements on Compressed Tablets.**—Specific surface area measurements were made on the tablets by the Brunauer–Emmett–Teller (B. E. T.) method (a low-temperature, nitrogen adsorption procedure) which was previously described in an application to surface area determinations of pharmaceutical powders (23). In the present procedure, several minor modifications were used according to the method of Swintosky and Rao (24); a sample tube was designed of slightly greater than $^3/_8$-in. internal diameter and without a constricted neck, permitting introduction of the intact tablets in a compact form and thus minimizing dead space in the tube. The form and dimensions of this sample tube are illustrated in Fig. 1.

**Density Measurements on Granules and Compressed Tablets.**—The hardness and disintegration properties of tablets obviously are influenced by the porosity or void space volume of the tablets. In order to determine the quantitative interrelationships between the variables, the void space of tablets formed under different compressional forces was calculated by evaluating the "true" and "apparent" densities of the tablets. True density of a tablet refers to the density of the compact, solid, mixed ingredients of which the tablet is composed, excluding the entire pore volume larger than spacings of molecular or atomic dimensions which exist in the crystal lattices of the solid ingredients. The apparent density of a tablet may be defined as the quotient of the weight and the geometric volume. Per cent porosity or void space may, in turn, be defined by the following expression:

$$\text{Per cent porosity} = \left(1 - \frac{\text{apparent density}}{\text{true density}}\right) 100$$

The true densities of granules and tablets employed in these studies were determined by means of a helium densitometer, described (1) in the first report of this series. A forthcoming publication (25) describes an improved model, its operation, and its applications to pharmaceutical problems.

The apparent volume of tablets may be calculated from measurements of their geometry or pycnometrically with mercury as a displacement liquid. In these studies, both methods were employed. A micrometer caliper was used for the measurement of geometric dimensions, whereas a specially designed and previously described (1) tablet pycnometer was employed in the mercury displacement method.

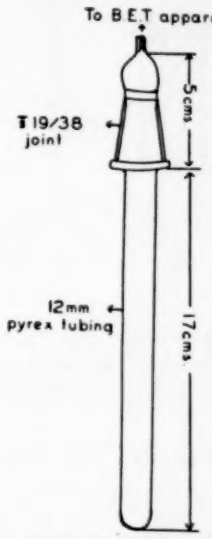


Fig. 1.—Sample tube for surface area determinations of tablets.

**Hardness Measurements on Compressed Tablets.**—Tablet hardness was determined by the use of a Strong Cobb Tablet Hardness Tester which has been mentioned by Hosler (16), and is illustrated diagrammatically in Fig. 2.

The tablet, *E*, was placed edgewise on the arm, *D*, of the tester. A pneumatic pressure, built up by pumping the handle, *A*, lowered the arm, *B*, toward the tablet. The handle was pumped gently until the force exerted on the tablet caused it to break. The reading on the recording scale was noted at the breakage force for the tablets prepared under variable compressional forces.

**Measurements on the Disintegration Time of Compressed Tablets.**—The method used in determining the disintegration time of tablets was essentially that of the U. S. P. (26) with a few minor modifications in operational design and procedure. The basket-rack portion of the disintegration test instrument contained four compartments instead of six recommended by the U. S. P. A four-liter beaker was used to contain the disintegration fluid water, which was maintained electrically at 36°. The basket-rack assembly was attached by a cord to a motor of variable speed. The number of revolutions of the motor, corresponding to the number of complete up-down cycles of the basket-rack assem-

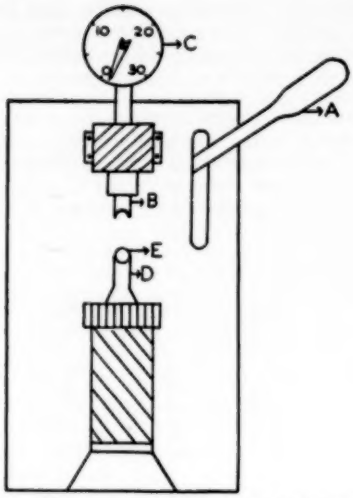


Fig. 2.—Diagrammatic sketch of the Strong Cobb Hardness Tester (front view). A—handle; B—upper arm; C—scale; D—lower arm; E—tablet.

bly, was kept at 31 cycles/min. The tablets were considered completely disintegrated when all of the disintegration products passed through the basket screen.

## RESULTS AND DISCUSSION

The influence of the maximal compressional force employed on the specific surface area of the tablets is illustrated graphically in Fig. 3. The specific surface area of the original granulation, corresponding to zero force in the figure, was 0.18 m.²/Gm. During the initial compressional process this value increased rapidly, indicating continual formation of new surfaces, probably through fragmentation of the primary crystalline constituent. Tablets produced with compressional force of 2,500 lb. contained particles with the largest specific surface area; their average diameter was one-fifth that of the primary particles making up the granulation, calculated from the fact that the specific surface area of the tablets produced under this force was 1.0 m.²/Gm. or little more than five times the area of the granulation. An increase in the compressive force beyond this point resulted in a progressive decrease in the effective specific surface area, probably resulting from cold bonding of the unit particles. The slight difference in the behavior of tablets of Batch 1 compared to tablets of Batch 2 toward changes in the compressive force (Fig. 3) is probably without significance, since the two curves are in good qualitative agreement.

The true densities of the tablets at 1,000 and 8,000 lb. compression and that of the original granulation as obtained on the helium densitometer are shown in Table I. The close agreement of the densities obtained for the granulation and for the tablets produced under 1,000 lb. of force indicates the ease with which helium penetrates the pores of these tablets. The density value obtained for tablets compressed with 8,000 lb. showed a very slight drop in density corresponding to approximately 0.3%, probably because of the inability of helium to penetrate completely into the pore spaces in the interior of the very highly compressed tablets. It is evident from these data that the helium densitometer is capable of yielding the true densities of granulations and even of normally compressed tablets because of the ability of helium gas to penetrate into minute pores and crevices.

Unlike true density, the apparent density of tablets is a sensitive function of the compressive force, as shown by the data represented in Fig. 4. There is a slight discrepancy between the pycnometric and geometric results, attributed to the tendency in the geometric method to subject the tablets to a slight degree of compression during

TABLE I.—TRUE DENSITIES OF SULFATHIAZOLE GRANULES AND TABLETS AT 30° DETERMINED BY HELIUM DENSITOMETRY (BATCH I)

| Sample | Wt. of Sample, Gm. (*M*) | Vol. of Sample, Cc. | Density, Gm./Cc. (*M*/*V*) |
|---|---|---|---|
| Sulfathiazole granules with 4.1% total starch | 9.0904 | 5.759 | 1.5785 |
| | 9.5297 | 6.040 | 1.5778 |
| Sulfathiazole tablets compressed at 1,000 lb. force | 9.7862 | 6.198 | 1.5789 |
| | 9.7772 | 6.194 | 1.5785 |
| Sulfathiazole tablets compressed at 8,000 lb. force | 11.4207 | 7.266 | 1.5718 |

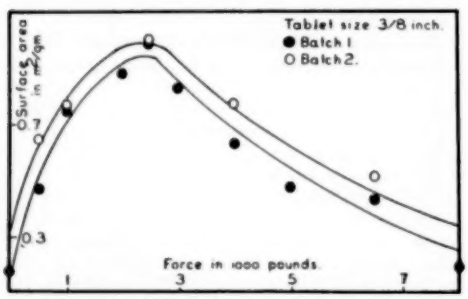


Fig. 3.—The effect of compressional force on the specific surface area of sulfathiazole tablets.

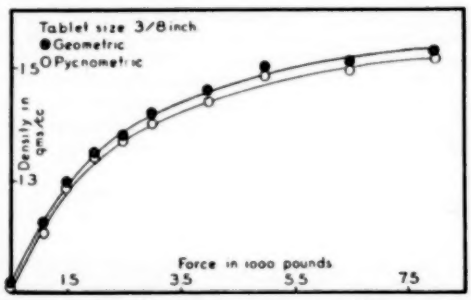


Fig. 4.—The influence of compressional force on the apparent density of sulfathiazole tablets.

measurement with a caliper. The logarithm of the force plotted against the degree of compression yields essentially a straight line except at the highest levels (Fig. 5). A similar relationship is evident in Fig. 6, where the porosities of the tablets have been plotted against the logarithms of the compressional force. It is interesting to note that even under 2,500 lb. of force, the porosity of these tablets is still as high as 9%. At the highest force level, the porosity is down to less than 3%.

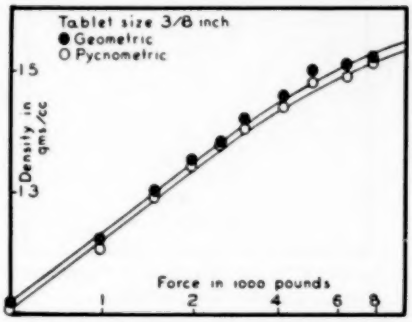


Fig. 5.—The influence of compressional force on the apparent density of sulfathiazole tablets.

These and other studies have shown that the porosity determinations are extremely reproducible and are sensitive only to the composition of the material under compression. Further studies may permit direct correlation of such data with molecular structure.

During the compressional process, a certain portion of the vertical thrust exerted by the moving punch is taken up by the die wall through a frictional mechanism. If an appreciable fraction of the total force is thus taken up, that part of the granulation near the bottom of the die cavity will be subjected to a proportionally lower degree of compression than the portion near the top. The data shown in Table II indicate that such is not the case. Tablets of different thickness prepared from 100, 200, 300, and 400 mg. of sulfathiazole granulation were found to have nearly identical densities when compressed to a given force level. If a significant portion of the compressive force were lost to the die wall, the thicker tablets should possess lower densities than the thinner ones because more die surface would be available for frictional transfer of the compressional force. For the tablets made under 4,000 lb., only the thinnest ones show any lowering in density. It therefore appears that the compressive force is uniform throughout the die cavity.

TABLE II.—APPARENT DENSITIES OF TABLETS MADE FROM DIFFERENT WEIGHTS OF GRANULES

| Weight of Granules, Mg. | 2,000 Lb. Force: Densities Individual Tablets, Gm./Cc. | 2,000 Lb. Force: Av. Tablet Density, Gm./Cc. | 4,000 Lb. Force: Densities Individual Tablets, Gm./Cc. | 4,000 Lb. Force: Av. Tablet Density, Gm./Cc. |
|---|---|---|---|---|
| 100 | 1.343 | 1.343 | 1.446 | 1.449 |
| | 1.342 | | 1.450 | |
| | 1.344 | | 1.458 | |
| | | | 1.445 | |
| | | | 1.446 | |
| | | | 1.448 | |
| 200 | 1.347 | 1.346 | 1.459 | 1.462 |
| | 1.346 | | 1.460 | |
| | 1.345 | | 1.468 | |
| | | | 1.456 | |
| | | | 1.465 | |
| | | | 1.461 | |
| 300 | 1.340 | 1.344 | 1.456 | 1.458 |
| | 1.344 | | 1.462 | |
| | 1.347 | | 1.453 | |
| | | | 1.458 | |
| | | | 1.461 | |
| 400 | 1.338 | 1.341 | 1.464 | 1.460 |
| | 1.343 | | 1.457 | |
| | | | 1.460 | |

The dependency of tablet hardness on the compressional force employed, determined on a Strong Cobb Hardness Tester, is shown in Fig. 7. The data represent the median value obtained with lots of five tablets for each force level. All the tablets were made and tested in one day. It appears from these results that the particle size distribution of granulations has only a limited effect, if any, on the hardness of the tablets. Batch 1 contained considerably more fine particles than Batch 2, yet the curves obtained are similar.

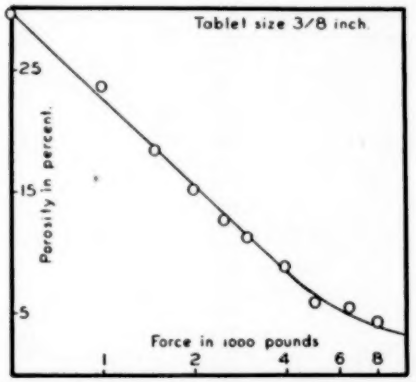


Fig. 6.—The effect of compressional force on the porosity of sulfathiazole tablets.

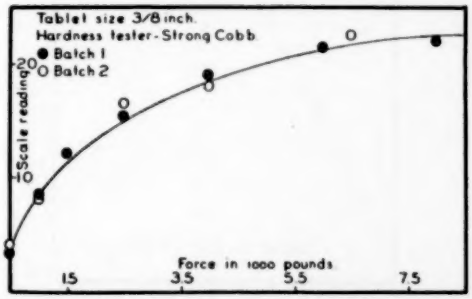


Fig. 7.—The effect of compressional force on the hardness of sulfathiazole tablets.

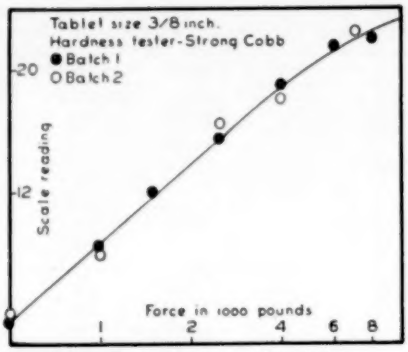


Fig. 8.—The effect of compressional force on the hardness of sulfathiazole tablets.

There appears to be a linear relationship between tablet hardness and the logarithm of maximal compressional force (Fig. 8). Up to a force level of 5,000 lb., a rather good straight line relationship is obtained; beyond this point, hardness increases less rapidly. An interesting linear correlation can also be found between Strong Cobb Hardness and the apparent density, or, equivalently, the porosity of the tablets. In Fig. 9, the hardness of sulfathiazole tablets compressed to different force levels has been plotted against their apparent density. A similar linear relationship is also found in a plot of porosity against hardness (Fig. 10).

It must be kept in mind in any fundamental attempt to correlate hardness with other variables that this term has a rather ambiguous meaning. In a general sense it has been accepted as a measure of the mechanical resistance of tablets to all sorts of attrition and breaking process. In practice, however, such distantly related characteristics as surface modulus, crushing tensile, impact tensile, and rate of wear of tablets have been used as measures of hardness. The Strong Cobb Hardness Tester measures only those qualities of tablets related to their resistance to crushing.

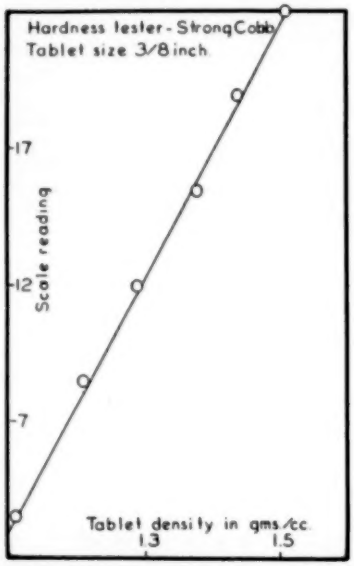


Fig. 9.—Plot of density *vs.* hardness of sulfathiazole tablets.

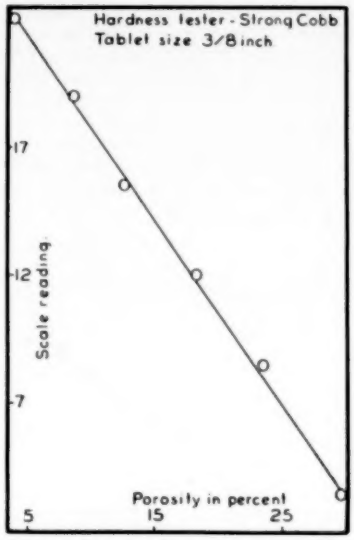


Fig. 10.—Plot of porosity against hardness of sulfathiazole tablets.

In Tables III, IV, V, and VI, results obtained from disintegration time studies on both Batch 1 and

TABLE III.—THE EFFECT OF COMPRESSIONAL FORCE ON THE DISINTEGRATION TIME OF SULFATHIAZOLE TABLETS (BATCH 1)

| Force, Lb. | Disintegration Time, Sec. | Median Reading, Sec. |
|---|---|---|
| | 50 | |
| | 55 | |
| 500 | 65 | 55 |
| | 65 | |
| | 50 | |
| | 110 | |
| | 115 | |
| 1,000 | 135 | 120 |
| | 120 | |
| | 140 | |
| | 195 | |
| | 195 | |
| 1,500 | 220 | 195 |
| | 195 | |
| | 310 | |
| | 265 | |
| | 290 | |
| 2,000 | 340 | 325 |
| | 335 | |
| | 325 | |
| | 600 | |
| | 695 | |
| 2,500 | 740 | 690 |
| | 690 | |
| | 710 | |

TABLE IV.—THE EFFECT OF COMPRESSIONAL FORCE ON THE DISINTEGRATION TIME OF SULFATHIAZOLE TABLETS (BATCH 1)

| Force, Lb. | Disintegration Time, Sec. | Median Reading, Sec. |
|---|---|---|
| | 45 | |
| | 55 | |
| 500 | 90 | 50 |
| | 35 | |
| | 50 | |
| | 150 | |
| | 145 | |
| 1,000 | 220 | 150 |
| | 125 | |
| | 215 | |
| | 200 | |
| | 255 | |
| 1,500 | 330 | 285 |
| | 285 | |
| | 295 | |
| | 480 | |
| | 525 | |
| 2,000 | 560 | 560 |
| | 655 | |
| | 970 | |
| | 1,070 | |
| | 1,180 | |
| 2,500 | 1,450 | 1,180 |
| | 1,110 | |
| | 1,530 | |
| | 11,300 | |
| 4,000 | 15,030 | 15,030 |
| | 19,130 | |

TABLE V.—THE EFFECT OF COMPRESSIONAL FORCE ON THE DISINTEGRATION TIME OF SULFATHIAZOLE TABLETS (BATCH 2)

| Force, Lb. | Disintegration Time, Sec. | Median Reading, Sec. |
|---|---|---|
| | 165 | |
| | 180 | |
| 500 | 205 | 180 |
| | 155 | |
| | 245 | |
| | 510 | |
| | 735 | |
| 1,000 | 830 | 735 |
| | 525 | |
| | 820 | |
| | 1,350 | |
| | 1,370 | |
| 1,500 | 1,720 | 1,370 |
| | 1,230 | |
| | 1,850 | |
| | 1,780 | |
| | 1,830 | |
| 2,000 | 4,170 | 1,830 |
| | 1,790 | |
| | 2,110 | |
| | 4,500 | |
| 2,500 | 4,990 | 4,990 |
| | 6,530 | |

Batch 2 are presented. The logarithms of the median values of the replicate runs have been plotted in Fig. 11 against the compressional force employed. The data appear to indicate that:

(*a*) The disintegration characteristics of tablets produced from any given granulation are reproducible.

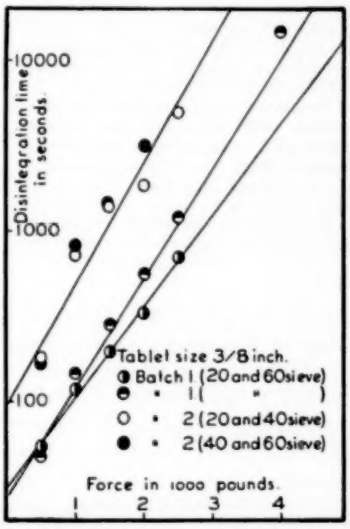


Fig. 11.—The effect of compressional force on the disintegration time of sulfathiazole tablets.

(*b*) Granulations produced from a given formulation at different times may exhibit different disintegrating characteristics.

(*c*) Granule size seems to have little or no influence on disintegration time in the particular ranges studied.

(*d*) There appears to be a good exponential relationship between disintegration time and compressional force.

It must be pointed out that these observations are valid only for the particular sulfathiazole formulation studied. A more extensive investigation is necessary to prove or disprove the validity of such observations for all types of formulations.

TABLE VI.—THE EFFECT OF COMPRESSIONAL FORCE ON THE DISINTEGRATION TIME OF SULFATHIAZOLE TABLETS (BATCH 2)

| Force, Lb. | Disintegration Time, Sec. | Median Reading, Sec. |
|---|---|---|
| | 175 | |
| | 180 | |
| 500 | 250 | 180 |
| | 180 | |
| | 265 | |
| | 660 | |
| | 785 | |
| 1,000 | 1,450 | 800 |
| | 800 | |
| | 1,050 | |
| | 835 | |
| 1,500 | 1,350 | 1,350 |
| | 1,830 | |
| | 2,750 | |
| | 3,170 | |
| 2,000 | 3,605 | 3,170 |
| | 3,130 | |
| | 3,500 | |

The present study is of a vertical type in that an intensive investigation has been made of a single formulation in an attempt to gather as much experimental information as possible for this one situation. Such an investigation permits more extensive correlation of the variables involved but leaves in doubt the general application of the conclusions. Horizontal extension of the present investigation over a large number of systems is necessary before the empirical relationships discussed can be of practical value, and such a study is under way.

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# The Influence of "Oral Antiseptics" on the Number of Bacteria Washed Out of the Mouth as Measured by a Rinse Technique*

By MORRIS OSTROLENK,† WILLIAM WEISS,‡ HOWARD EDELSON,‡ and STANLEY FRIEDBERG†

**Hourly rinses of 20 ml. each of physiological salt solution for thirty seconds established a pattern of fluctuation in the number of bacteria in the mouth during the course of an eight-hour working day. There was a gradual increase in the number of bacteria during the first three hourly washes. The influence of eating, between the third and fourth hours, was reflected in a sharp reduction in the number of bacteria recovered at the fourth hourly wash. The number of bacteria increased at the fifth hourly wash (first hour after lunch) and continued to increase at each succeeding wash. The original prelunch levels in bacterial count were reached by approximately the seventh hour (third hour after lunch). Subsequent tests with "oral antiseptics" were made, employing four rinses at hourly intervals in order to avoid the complicating influence of eating. The results of these tests are compared with the levels indicated to be normal by physiological salt solution rinses over the same test periods.**

IT IS now generally recognized that the *in vitro* test procedures frequently used for comparing the activity of drugs represented as antiseptics are not accurate or adequate measures of the efficacy of such products as antibacterial agents. The need for test procedures capable of revealing the efficacy of such products under actual conditions of use has been pointed out (1, 2). The present paper is a progress report on an organized study designed to evaluate the efficacy of that class of drug products represented as oral antiseptics.

The problem of determining the numbers and types of bacteria normally present in the oral cavity is difficult. It is obvious that the numbers

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and types of microorganisms recoverable from the oral cavity will vary with the method employed for securing representative samples for analysis. So divergent are the quantitative results obtained by the use of paraffin (3), swabs (4), toothbrushes (5), rinses (6), and direct examination of saliva (7) that comparisons are virtually impossible.

For a practical approach, the method of obtaining oral cavity samples for bacteriological analysis should be associated with the intended use of the class of products under test. It should be possible to correlate the efficacy of a medicated mouthwash with an innocuous oral rinse, toothpastes with brushing alone, germicidal gums with the chewing of paraffin or some similar substance, and medicated lozenges with the corresponding effect of an equal weight and volume of the lozenge minus the medicinal ingredient. The remaining factors involved in evaluating the antibacterial effect of this type of product, such as the types of bacterial culture including such items as media, time and temperature of incubation, and methods of calculating numbers of bacteria, can more readily be solved.

Data presented in a previous publication (8) demonstrated that the use of 20 ml. of 0.85 per cent salt solution for thirty seconds should be adopted as the oral rinse technique.

## MATERIALS AND METHODS

The commercial products employed in the tests were purchased in retail stores in Washington, D. C., and are identified by a code letter. Product D is antiseptic solution N. F. Although the products were chosen to represent a variety of primary active ingredients, D, F, and H are of the same general composition.

Each product was tested by the conventional *in vitro* loop technique (9) at 37° against 15 test organisms at the strongest concentration recommended for use with exposure periods ranging from thirty seconds to fifteen minutes. Each volunteer rinsed his mouth with 20 ml. of physiological salt solution for thirty seconds. Rinse solutions were collected in sterile Petri dishes and 1-ml. aliquots were immediately diluted with sterile 0.85% salt solution. These suspensions were shaken vigorously for ten seconds and planted in semisolid thioglycollate shake agar (U. S. P. fluid thioglycollate medium) with an increased agar content of 0.4% (8), blood agar (4% human blood), and streptococcus agar (10). Tubes and plates were incubated at 34° for forty-eight hours. Bacterial counts were calculated on the basis of the entire rinse volume (20 ml.).

## EXPERIMENTAL

**In Vitro Tests.**—With the exception of product C, products possessed equal antibacterial activity. With product C, periods ranging from three to fifteen minutes were required to destroy the test organisms, whereas the other test products killed the organisms within thirty seconds. On the basis of these tests, all of the products, except possibly C, possessed equal antibacterial activity.

**In Vivo Experiments.**—Prior studies (8) have shown that the numbers of bacteria recovered from the mouths of human subjects by the rinse method are significantly lower in the afternoon than in the morning due to mechanical degerming from eating the noon meal.

Preliminary experimentation was conducted to measure the extent of this effect and to select the optimum period for testing the effect of commercial products on oral bacteria.

Ten subjects were examined at hourly intervals for seven hours on each of three days. All subjects ate lunch approximately one-half hour after their third rinse. The results are summarized in Table I and Fig. 1, and show that the numbers of bacteria rinsed from the oral cavity tend to increase about 20% during the rinses prior to lunch. There is a

TABLE I.—THE INFLUENCE OF LUNCH ON THE NUMBER OF BACTERIA RINSED FROM THE MOUTH

| Time before (−) and after (+) Lunch | Average Bacterial Count in Millions[a] | | |
|---|---|---|---|
| | Thioglycollate Agar | Blood Agar | Strep. Agar |
| −2 Hr. 30 min. | 127 | 130 | 71 |
| −1 Hr. 30 min. | 129 | 134 | 72 |
| −30 Min. | 153 | 160 | 88 |
| +30 Min. | 68 | 57 | 28 |
| +1 Hr. 30 min. | 107 | 117 | 59 |
| +2 Hr. 30 min. | 126 | 145 | 72 |
| +3 Hr. 30 min. | 153 | 165 | 88 |

[a] Experiment includes 10 subjects, each repeated three times.

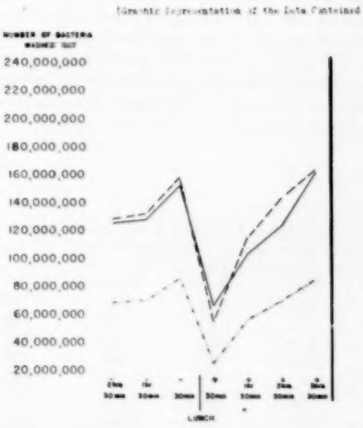


Fig. 1.—The influence of lunch on the number of bacteria in the mouth (graphic representation of data contained in Table I).

———Thioglycollate agar. – – –Blood agar. –·–·–·–Streptococcus agar.

TABLE II.—EXPERIMENTAL DESIGN

| Subject | Group | Day Examined | Schedule of Mouthwash Tested, Week | | | |
|---|---|---|---|---|---|---|
| | | | 1st | 2nd | 3rd | 4th |
| 1 | 1 | Monday | D | X | C | B |
| 2 | 1 | Monday | X | D | B | C |
| 3 | 1 | Monday | C | B | D | X |
| 4 | 1 | Monday | B | C | X | D |
| 5 | 2 | Tuesday | E | X | G | F |
| 6 | 2 | Tuesday | F | G | X | E |
| 7 | 2 | Tuesday | X | E | F | G |
| 8 | 2 | Tuesday | G | F | E | X |

sharp reduction in the bacterial counts after lunch to about half or less than the counts just prior to lunch, followed by a progressive increase to the pre-lunch level at the fourth rinse after lunch.

The morning period was selected as the time interval for conducting further studies on a pattern of mouth washings as follows: the subjects rinsed their mouths with 20 ml. of physiological salt solution for thirty seconds at hourly intervals for four hours. At the thirty-minute point between the second and third washes, each subject received either 20 ml. of a commercial mouth wash or an equal volume of 0.85% salt solution as a control.

No restrictions were made on normal habits of drinking or smoking. However, none of the subjects ate solid foods nor chewed gum during the course of the experiments. The dilutions of mouthwash employed were the maximum concentrations recommended on the labels of the test products.

The tests were conducted during the spring and summer of 1952. The eight subjects available at the start of the experiment were divided into two equal groups, each tested in the same manner with respect to the compounds and the control wash. Group 1 was tested on successive Mondays and Group 2 on Tuesdays. Compounds B, C, and D were used with Group 1 and compounds E, F, and G with Group 2 so that any differences in the effect of the compounds within each group would be independent of the differences due to the subjects. The design, a Latin square, allowed for the testing of each compound and the control on the same day to eliminate any time trend (Table II). This four-week test pattern was repeated three times for a total of twelve weeks. The salt solution control, X, measured the relationship between the compounds in the two groups.

The experimental design was not followed rigidly with one of the subjects due to unavoidable circumstances. Subject No. 5 followed the schedule for six weeks, was absent for six weeks, and then continued the test to the completion of the schedule. To accomplish this, the latter tests were made over a two-week period with not more than one test wash per day.

A ninth subject (No. 15) was started when subject No. 5 dropped out of the experiment. Tests on this subject were made for ten consecutive weeks with two additional tests on different days during the eighth and tenth weeks. Results are shown in Tables III, IV, and V, and Figs. 2, 3, and 5.

In a separate experiment, a tenth subject was tested in the afternoon on compounds designated E′, H, and X.

## RESULTS

An analysis of the data reveals that on the basis of the first count washes for thioglycollate, blood, or streptococcus agar, there are significant differences in the bacterial levels of the test subjects. The average of the 12 first rinse counts on thioglycollate agar for each subject ranged from a low of 40 million to a high of 281 million (Table III). This difference in bacterial counts between subjects is of considerably greater magnitude and occurs more consistently than the weekly variations for any one individual. Regardless of the magnitude of the initial bacterial counts per individual, as measured by thioglycollate or blood agar, the consistent productivity of streptococcus agar was approximately 55% of either of these media, and the same relationship prevails for the results of the second wash.

TABLE III.—AVERAGE BACTERIAL COUNTS FOR EACH SUBJECT BY MEDIA IN THE 1ST AND 2ND WASHES[a]

| Subject | 1st Wash | | | 2nd Wash | | | 1st and 2nd Wash | | |
|---|---|---|---|---|---|---|---|---|---|
| | Thio. | Blood | Strep. | Thio. | Blood | Strep. | Thio. | Blood | Strep. |
| 1 | 164 | 176 | 101 | 136 | 126 | 76 | 150 | 149 | 88 |
| 2 | 72 | 69 | 37 | 91 | 101 | 46 | 81 | 84 | 41 |
| 3 | 281 | 252 | 150 | 267 | 251 | 144 | 274 | 251 | 147 |
| 4 | 132 | 153 | 93 | 116 | 154 | 81 | 123 | 153 | 87 |
| 5 | 73 | 71 | 39 | 115 | 122 | 64 | 92 | 93 | 50 |
| 6 | 148 | 188 | 75 | 151 | 171 | 86 | 150 | 180 | 80 |
| 7 | 65 | 67 | 34 | 68 | 81 | 39 | 67 | 74 | 37 |
| 8 | 40 | 47 | 25 | 94 | 110 | 58 | 62 | 72 | 38 |
| 15 | 56 | 53 | 41 | 54 | 60 | 40 | 55 | 57 | 40 |
| All | 96 | 100 | 56 | 110 | 121 | 65 | 103 | 110 | 60 |

[a] Average of 12 tests per subject on each medium, bacterial counts in millions. The combined averages of all the subjects are calculated from the logarithms of their individual counts.

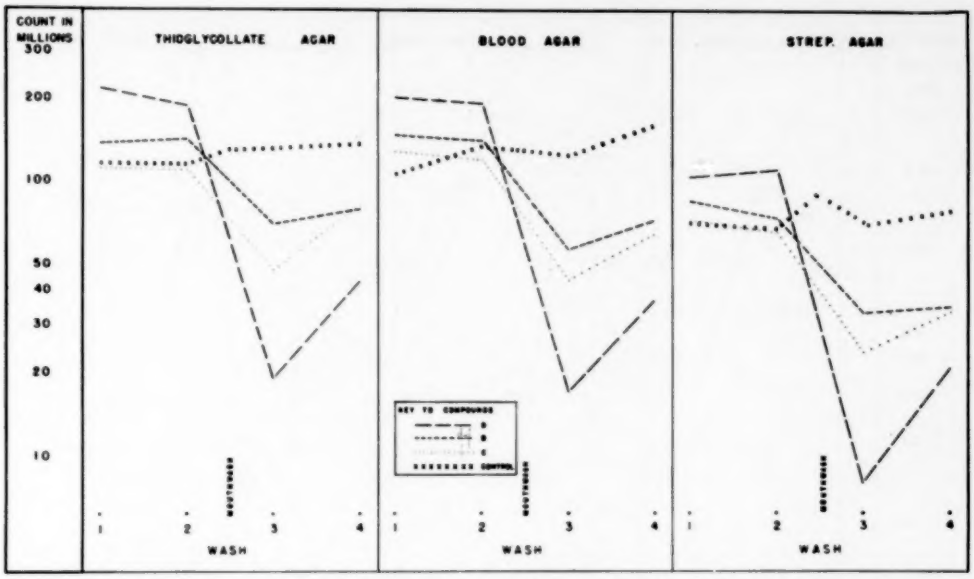


Fig. 2.—The influence of antiseptic mouthwash on the number of bacteria in the oral cavity.

Although there appears to be an over-all increase in the bacterial counts from the first to the second wash, such increases are not consistent with every individual. The subjects may be arbitrarily classified into one of three groups, based on their responses to the first and second washes: (*a*) those who show a consistent increase; (*b*) those who show a consistent decrease; (*c*) those who do not show a consistent trend.

In order to establish a base from which to measure the effect of the products, the results of the third rinse (thirty minutes after use) were compared with the results of the second rinse and the average of the first and second rinses. The measure of accuracy of the base was the consistency of results of the individual subject given a specific test product. Statistical analysis showed that either base gave approximately the same degree of precision and that the

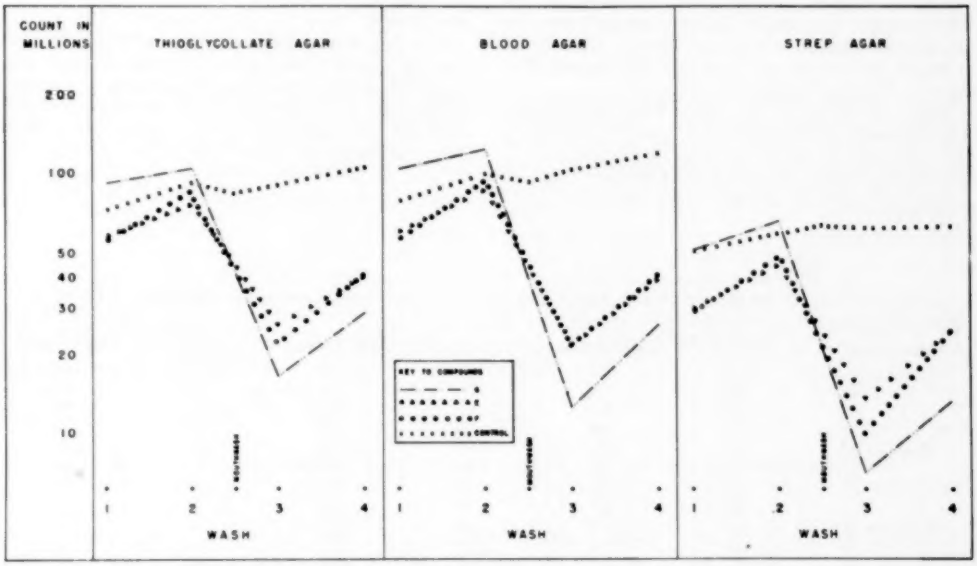


Fig. 3.—The influence of antiseptic mouthwash on the number of bacteria in the oral cavity.

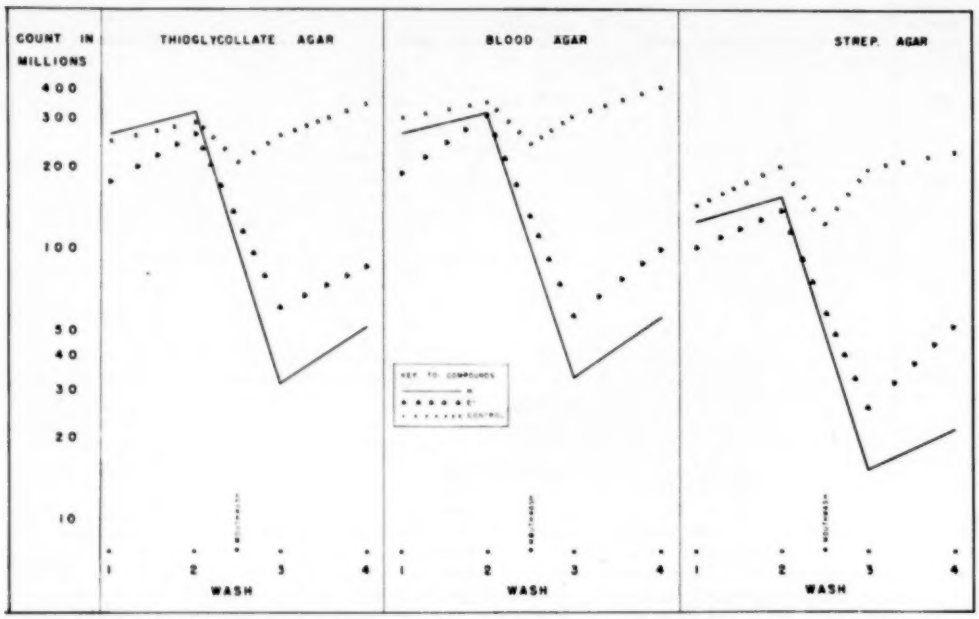


Fig. 4.—The influence of antiseptic mouthwash on the number of bacteria in the oral cavity.

average per cent recoveries for each compound were approximately the same by both methods of calculation. As a consequence, the results (Table IV), showing the effect of the several test products expressed as per cent recoveries thirty minutes and one hour and thirty minutes after use of each product, are the ratios of rinses 3 and 4 compared to rinse 2.

Figures 2, 3, and 4 illustrate the combined results for all the subjects on each of the compounds tested. The average number of bacteria washed out, expressed in millions, is represented on a logarithmic scale for each of the test intervals (before and after the use of a mouthwash).

Figure 2 represents the results with four subjects who received mouthwashes B, C, and D, and 0.85% salt solution, X, as a control. Each subject received four designated mouthwashes three times, in accordance with the schedule shown in Table II.

Similarly, in Fig. 3 are shown the results with the five subjects in which preparations E, F, G, and X were employed, and in Fig. 4 those with subject 10 tested with a control wash, X, and two commercial solutions H and E′.

It is noteworthy that for all of the products and the subjects, the percentage recoveries (thirty minutes after use of the mouthwashes) on thioglycollate agar (37%) were consistently higher than on blood agar (30%) or streptococcus agar (31%); see Table V.

It is apparent from inspection of Table IV and Figs. 2, 3, and 4 that the seven products vary substantially in their effect on the number of bacteria recoverable from the mouth by the rinse method, although they appear to be of comparable activity from the results of the *in vitro* tests. Products D, G, and H produced reductions in the number of bacteria to a range of 10–14% thirty minutes after

TABLE IV.—COMPARISON OF THE PER CENT RECOVERIES OF THE SEVERAL COMPOUNDS TESTED

| Compound | Thioglycollate Agar | | Blood Agar | | Streptococcus Agar | |
|---|---|---|---|---|---|---|
| | 30 Min. | 1 Hr. 30 Min. | 30 Min. | 1 Hr. 30 Min. | 30 Min. | 1 Hr. 30 Min. |
| Control X | 115 | 120 | 93 | 119 | 104 | 112 |
| B | 43 | 74 | 36 | 54 | 36 | 51 |
| C | 49 | 55 | 40 | 51 | 46 | 48 |
| D | 12 | 23 | 9 | 19 | 7 | 19 |
| Control X | 99 | 115 | 101 | 116 | 106 | 107 |
| E | 35 | 52 | 25 | 47 | 31 | 56 |
| F | 26 | 49 | 23 | 44 | 21 | 51 |
| G | 14 | 26 | 10 | 21 | 10 | 20 |
| Control X | 90 | 117 | 87 | 112 | 97 | 114 |
| H | 10 | 16 | 10 | 17 | 10 | 14 |
| E′ | 23 | 32 | 18 | 32 | 18 | 37 |

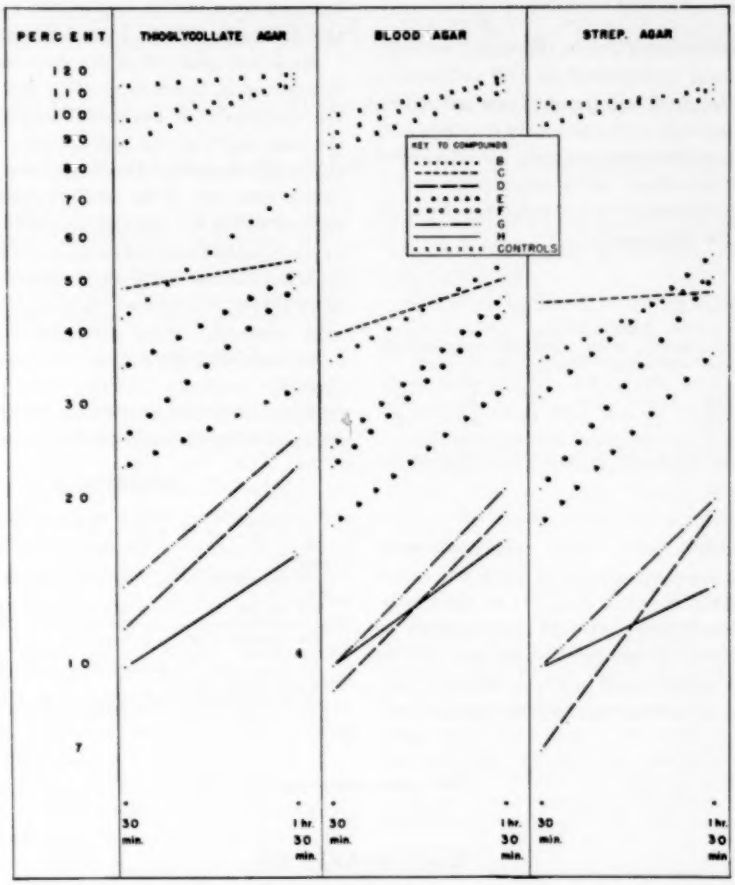


Fig. 5.—Per cent recoveries in thirty minutes and one hour thirty minutes after the use of test compounds.

use and to a range of 16–26% one hour and thirty minutes after use. In contrast, products B and C produced reductions of 43 and 49%, respectively, thirty minutes after use and 74 and 55% one hour and thirty minutes after use.

It is of interest that whereas products B and C gave approximately the same ranges of activity, their chemical compositions are vastly different. Similarly, products E and F, which show no appreciable differences within the limits of the test, are unrelated chemically.

It is evident from these data and from inspection of Fig. 5 that the number of bacteria increase with time after use of the test products, the rate varying with the product. It is unfortunate that these increases could not be followed by successive rinses to elicit more information on the time required to reach original levels. Detailed data on the reductions by subject are not presented. Despite fluctuations in the results from subject to subject in average per cent recoveries, it is clearly evident that several of the products tested differ in their activity.

With preparations E and G, tested on 5 subjects, the average per cent recoveries of microorganisms on thioglycollate agar after thirty minutes are shown in Table VI.

TABLE V.—PER CENT RECOVERIES THIRTY MINUTES AFTER THE USE OF TEST COMPOUNDS

| Medium | Subject Number | | | | | | | | | Av. |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 15 | |
| Thioglycollate agar | 47 | 43 | 33 | 44 | 38 | 39 | 18 | 32 | 50 | 37 |
| Blood agar | 30 | 34 | 31 | 37 | 31 | 36 | 14 | 24 | 43 | 30 |
| Streptococcus agar | 25 | 40 | 30 | 40 | 36 | 34 | 13 | 30 | 42 | 31 |

## CONCLUSIONS

The procedure employed in this study for comparing products represented as oral antiseptics, under conditions of use, reveals marked differences in the activity of the products not shown by conventional *in vitro* test methods. It is believed that the method offers useful information on the degree of antibacterial activity exerted by such products in the oral cavity.

TABLE VI

| Compound | Subject Number 5 | 6 | 7 | 8 | 15 | Av.[a] |
|---|---|---|---|---|---|---|
| E | 49 | 43 | 18 | 27 | 51 | 35 |
| G | 15 | 15 | 9 | 15 | 20 | 14 |

[a] Averages calculated from the logarithms of the bacteria counts.

These studies have been concerned with quantitative measurements and have not taken into account the qualitative effects on the microflora of the oral cavity. While the similarity of the results with the products under test on the three culture media used tend to indicate that selectivity of action against various types of oral bacteria may not be important, explorations of the qualitative effects are indicated.

Statistical analysis of the data shows that the differences in product activity are unrelated to the magnitude of the initial bacterial counts of the oral cavity of the test subject. It is evident that large numbers of bacteria remain in the oral cavity after use of the test products, the magnitude of which will depend on the initial numbers in the mouth of the test subject. It is also evident that individuals with high normal counts may carry larger numbers of bacteria after treatment with relatively active products than untreated individuals with low counts. In the final analysis, therefore, evaluation of the efficacy of products represented as oral antiseptics requires the application of carefully controlled clinical studies.

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## WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Norepinephrine
Glycyl-L-phenylalaninamide
2,5-Dimethylthiophene
Furocoumarin
Benzoyl-L-(+)-alanine
2-Formyl-3-carboxylpyridine
*n*-Pentacosanic acid
7-Hydroxy-2-naphthoic acid
2,3,4-Trichlorophenol
Glucosone
Camphoquinone
5,6-Dimethylbenzimidazole
3-Aminothianaphthene
Scopine
$\alpha,\alpha$-Diphenyl-$\beta$-methyl fulgide
Pyrimidine
2-Nitro-4-aminophenol
Mellitic acid
Oxine-5-carboxylic acid

# Esters of Gentisic Acid and Their Toxicities*,**

## I. Preparation and Analyses of New Esters of Gentisic Acid

By J. F. NASH,† F. W. BOPE,‡ and B. V. CHRISTENSEN§

**The syntheses of new esters of gentisic acid are discussed. Eight esters of gentisic acid and amino alcohols have been prepared by the interaction of dialkylaminoalkyl chlorides and gentisic acid. Five esters of gentisic acid and other carboxylic acids are prepared by the interaction of acid chlorides and gentisic acid in alkaline media.**

ALTHOUGH salicylic acid and sodium salicylate have been used in the treatment of rheumatic conditions since 1876 and 1877, respectively (1), the metabolite of salicylic acid, gentisic acid, remained unknown medicinally until 1948 when Meyer and Ragen reported the use of sodium gentisate for the relief of rheumatic symptoms (2). Since then, numerous reports from the Western Hemisphere have substantiated the original belief of Meyer and Ragen.

The specific effect of salicylates upon the activity of hyaluronidase and salicylate action in rheumatic conditions dates back to the work of Guerra in 1946 (3, 4).

Meyer and Ragan (2, 5) claimed that the inhibition of hyaluronidase activity was due to the fact that the salicylates are converted to gentisic acid which acts as the true inhibitor. Salicylates are known to yield from 4 to 8 per cent gentisic acid in the body (1, 6, 7).

However, Roseman, *et al.* (8), showed that analytically pure gentisic acid is devoid of hyaluronidase inhibitory activity, while crude gentisic acid, as well as pure gentisic acid, treated with alkali in the presence of air possesses activity. Therefore, it was concluded that the activity of gentisic acid was probably due to impurities in the product which had been formed by oxidation.

Another cause of rheumatoid arthritis has been thought to be hormone imbalance (6). The salicylates may stimulate the adrenal cortex, causing liberation of croticoid substances. Adrenal cortical stimulation can be demonstrated in rats and guinea pigs by an increase in secretion of urinary 12-ketosteroids and a decrease in circulating eosinophils (9).

The work undertaken by the authors was the preparation of new esters of gentisic acid. It was believed that derivatives of gentisic acid may act more favorably in the treatment of rheumatism or as analgesics than gentisic acid itself. Since amino alcohols are components of other medicinally useful drugs, esters of gentisic acid and amino alcohols were prepared. Esters of gentisic acid and various other carboxylic acids were also prepared.

### EXPERIMENTAL

**Preparation of Esters of Gentisic Acid and Amino Alcohols.**—Esters of gentisic acid and amino alcohols were prepared by the Horenstein and Pahliche procedure (10). The process involves the condensation of a carboxylic acid and a chloralkamine in equimolar quantities either with or without a solvent. The best yield was generally obtained by dissolving the acid in isopropyl alcohol, adding the halide and refluxing the mixture for several hours. The hydrochloride generally precipitated when the solution was cooled. If the product remained in solution the solvent was removed under reduced pressure and the mass was treated with a solvent in which the salt was insoluble. The compound usually separated as a white solid and was then recrystallized from a suitable solvent.

The dry method consisted of mixing the halide with an alcoholic solution of the carboxylic acid, removing the alcohol under reduced pressure, and heating the residue ten hours at 90–100°. The mass was then extracted with a suitable solvent such as acetone or ethyl acetate to remove the unreacted materials. The insoluble salt of the amine was filtered and recrystallized from a suitable solvent.

The chloralkamines were obtained by the reaction of 2-diethylaminoethanol or 3-diethylamino-1-propanol with thionyl chloride. The reaction yielded the hydrochlorides. The salts were dissolved in cold water, and the solution was made strongly alkaline and extracted several times with ether. After removal of the ether, the chloralkamines were distilled as colorless, strongly ammoniacal-smelling liquids (11, 12) in about 60% yield.

The properties and analyses of the hydrochlorides of the new esters of gentisic acid and amino alcohols are shown in Table I. The molecular weights were calculated from the neutralization equivalents of the bases. These equivalents were determined by adding an excess of standard base to 25 ml. of a 50% alcoholic solution of the amine hydrochloride, and titrating the excess alkali and the amine with stand-

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TABLE I.—PROPERTIES AND ANALYSIS OF ESTERS OF GENTISIC ACID AND AMINO ALCOHOLS



| R | R′ | Empirical Formula | M. P.[a] | Molecular Wt. Calcd.[b] | Molecular Wt. Found[c] | Analysis for Nitrogen Calcd. | Analysis for Nitrogen Found[c] | Analysis for Chlorine Calcd. | Analysis for Chlorine Found[c] | Analysis for Carbon and Hydrogen Calcd. | Analysis for Carbon and Hydrogen Found | Yield, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| —$C_2H_4N(C_2H_5)_2\cdot HCl$ | H— | $C_{13}H_{20}O_4NCl$ | 150–151° | 253.30 | 252.11 | 4.84 | 4.68 | 12.58 | 12.61 | ... | ... | 19.8 |
| —$C_2H_4N(C_2H_5)_2\cdot HCl$ | $CH_3CO$— | $C_{15}H_{22}O_5NCl$ | 133.5–134.5° | 295.33 | 301.65 | 4.22 | 4.17 | 10.69 | 10.62 | C, 54.30<br>H, 6.68 | C, 54.41<br>H, 6.78 | 44.2 |
| —$C_2H_4N(C_2H_5)_2\cdot HCl$ | $CH_3$— | $C_{14}H_{22}O_4NCl$ | 182.3–183.0° | 267.32 | 269.35 | 4.61 | 4.70 | 11.68 | 11.62 | ... | ... | 58.0 |
| —$C_2H_4N(C_2H_5)_2\cdot HCl$ | $C_6H_5CO$— | $C_{20}H_{24}O_5NCl$ | 153–154° | 357.39 | 355.75 | 3.56 | 3.42 | 9.00 | 8.94 | C, 60.85<br>H, 6.14 | C, 60.40<br>H, 6.41 | 53.1 |
| —$C_3H_6N(C_2H_5)_2\cdot HCl$ | H— | $C_{14}H_{22}O_4NCl$ | 171.5–172.5° | 267.32 | 265.50 | 4.61 | 4.65 | 11.68 | 11.80 | C, 55.35<br>H, 7.30 | C, 55.45<br>H, 7.42 | 24.5 |
| —$C_3H_6N(C_2H_5)_2\cdot HCl$ | $CH_3CO$— | $C_{16}H_{24}O_5NCl$ | 157–157.5° | 309.35 | 309.46 | 4.05 | 4.04 | 10.25 | 10.34 | ... | ... | 20.6 |
| —$C_3H_6N(C_2H_5)_2\cdot HCl$ | $CH_3$— | $C_{15}H_{24}O_4NCl$ | 184.5–185.3° | 281.34 | 280.17 | 4.41 | 4.35 | 11.16 | 11.27 | ... | ... | 48.1 |
| —$C_3H_6N(C_2H_5)_2\cdot HCl$ | $C_6H_5CO$— | $C_{21}H_{26}O_5NCl$ | 142.7–143.7° | 373.42 | 366.84 | 3.43 | 3.60 | 8.69 | 8.62 | ... | ... | 16.9 |

[a] Uncorrected.
[b] Calcd. for free amine.
[c] Average of two values.

ard acid, using the Beckman Model G *p*H meter to determine the end point.

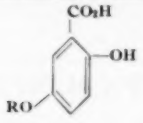
The hydrochlorides of the amino alcohol esters of gentisic acid are readily soluble in water but insoluble in organic solvents. However, aqueous solutions of the salts must be buffered so that the solution is acid to insure stability.

**Preparation of Esters of Gentisic Acid and Other Carboxylic Acids.**—Esters of gentisic acid and other carboxylic acids were prepared by a Schotten-Baumann reaction. Equimolar portions of gentisic acid and various acid chlorides were reacted in 10% sodium hydroxide. Gentisic acid was dissolved in the alkaline solution and the acid chloride was added with continuous stirring. The mixture was cooled slowly to room temperature and then below by an ice bath. Cold dilute hydrochloric acid was added until the mixture was strongly acid. The resulting precipitate was filtered and dried. The product was purified by dissolving it in acetone or alcohol and reprecipitating by addition of water. Final purification was brought about by recrystallization from a suitable solvent.

Four of the acid chlorides were prepared in the usual manner employing an excess of thionyl chloride. The acid chloride of succinic acid was prepared by the interaction of equimolar quantities of the acid and phosphorus pentachloride. The mixture was heated to 125° for twenty hours, the phosphorus oxychloride was removed, and the acid chloride was distilled.

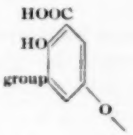
The properties and analyses of the esters of gentisic acid and other carboxylic acids are shown in Table II. These esters can be rendered partially soluble by making the sodium salt of the free carboxyl group. Although the salts may be unstable in solution, they are stable as solids. Neutralization equivalents of these esters were determined in the usual manner, and all esters gave positive tests with ferric chloride test solution, indicating a free phenolic hydroxyl group present.

TABLE II.—PROPERTIES AND ANALYSES OF ESTERS OF GENTISIC ACID AND OTHER CARBOXYLIC ACIDS



| RO— | Empirical Formula | M. P.[b] | Molecular Wt. Calcd. | Molecular Wt. Found[c] | Analysis for Carbon and Hydrogen Calcd. | Analysis for Carbon and Hydrogen Found | Yield, % |
|---|---|---|---|---|---|---|---|
| $C_6H_5CH_2CO$— | $C_{15}H_{12}O_5$ | 145.5–146.5° | 272.25 | 273.38 | C, 66.17<br>H, 4.44 | C, 66.21<br>H, 4.51 | 28.3 |
| 4-$(NO_2)C_6H_4CO$— | $C_{14}H_9O_7N$ | 229–230.5° d. | 303.20 | 304.19 | C, 55.46<br>H, 2.99 | C, 55.43<br>H, 2.97 | 33.0 |
| 4-$(CH_3O)C_6H_4CO$— | $C_{15}H_{12}O_6$ | 208.4–209.5° | 288.25 | 290.50 | C, 62.50<br>H, 4.19 | C, 62.43<br>H, 4.54 | 66.6 |
| 2-$(CH_3CO_2)C_6H_4CO$—[a] | $C_{16}H_{12}O_7$ | 203.5–204.7° | 316.26 | 285.23 | C, 60.76<br>H, 3.83 | C, 61.63[e]<br>H, 3.89[e] | 16.1 |
| $C_7H_5O_4$[d]$OCC_2H_4CO$— | $C_{18}H_{14}O_{10}$ | 230–231.5° d. | 390.29 | 388.37 | C, 55.39<br>H, 3.62 | C, 55.20<br>H, 3.82 | 10.1 |

[a] Doubtful formula.
[b] Uncorrected.
[c] Average of two values.
[d] $C_7H_5O_4$ is a second gentisic acid group



## SUMMARY AND CONCLUSIONS

1. Thirteen new esters of gentisic acid were prepared: eight are esters of gentisic acid and amino alcohols; five are esters of gentisic acid and other carboxylic acids.

2. Amino alcohol esters were prepared in good yield by the interaction of gentisic acid and chlorides of 2-diethylaminoethanol and 3-diethylamino-1-propanol. All compounds were analyzed for nitrogen and chlorine, and three were analyzed for carbon and hydrogen. A modification of the usual procedure for determination of neutralization equivalents of the hydrochlorides of the esters was employed.

3. Esters of gentisic acid and other carboxylic acids were prepared by the interaction of the various acid chlorides and the number 5 hydroxyl group of gentisic acid in alkaline media, i. e., Schotten-Baumann reaction. All the esters were analyzed for carbon and hydrogen.

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# A Buffer System for Ophthalmic Solutions of Sodium Sulfonamides*

By HARRY B. KOSTENBAUDER,† FRED B. GABLE,† and ALFRED N. MARTIN‡

**An investigation has been made to determine whether a chemical incompatibility exists between the sulfonamides and borate buffers, since these sulfonamide salts in concentrations of 5% are not soluble at *p*H 8.6. The results of the study indicate that the salts of sulfadiazine and sulfathiazole are not incompatible with borate buffers. At a *p*H of 8.6, it was found that only about 2% concentration of sodium sulfathiazole and 1% concentration of sodium sulfadiazine may be attained in any buffer, regardless of chemical species, whereas buffers which provide a higher *p*H are required for 5% solutions of these sulfonamides. A formula for a buffer is suggested to replace the previously used vehicle of *p*H 8.6. Solutions of sodium sulfonamides are subject to color changes and mold growth during shelf life, and the use of certain stabilizers to overcome these features is discussed.**

IN RECENT YEARS a borate buffer system at *p*H 8.6 has been suggested as a vehicle for ophthalmic solutions of sulfathiazole sodium and sulfadiazine sodium in concentrations of 5 per cent. Pharmacists have noted, however, that 5 per cent concentrations of these salts are not soluble in the buffer.

The present investigation was undertaken to formulate a suitable buffer solution for use in the preparation of ophthalmic solutions of the sodium sulfonamides. The investigation involved considerations of chemical compatibility of the buffer system and the drug, the most desirable *p*H range, and preservation of the solutions.

Chemical incompatibility between borates and the sulfonamides was ruled out in preliminary work which indicated that the salts were soluble in the borate buffer if the *p*H was sufficiently high. The *p*H of 5 per cent aqueous solutions of these products is quite high (9.3 for sulfadiazine sodium, 9.7 for sulfamerazine sodium, 9.8 for sulfathiazole sodium, and 10.9 for sulfapyridine sodium[1]).

To avoid discomfort when ophthalmic solutions are instilled in the eye, it is advisable to adjust the *p*H of the solution to a value as near to that of the lacrimal fluid as practical for stability of the solution. A survey of the literature failed to reveal solubilities of the sulfonamides as a function of *p*H in the high *p*H ranges which are necessary if the drugs are to be used in the concentrations required for ophthalmological therapy.

The modified buffer equation has been widely used in solubility calculations to obtain the *p*H of various concentrations of salts, or conversely, to calculate the total solubility of the substance at any given *p*H value. The equation for a salt of a weak acid is

$$pH = pK_a + \log \frac{S}{[HA]} \qquad (1)$$

in which $pK_a$ is the negative logarithm of the ionization constant of the weak acid, S is the maximum equilibrium concentration of the salt in a saturated solution of the weak acid, and [HA] is the molar solubility of the weak acid in water. Krebs and Speakman (1) employed a form of this equation to calculate the solubility of sulfonamides as a function of *p*H at 37° but did not publish data for values higher than *p*H 7.85.

For each sulfonamide, $pK_a$ and [HA] are constants at a given temperature; thus, Eq. 1 may be rewritten as

$$pH = pK_a + \log \frac{10 \times \%}{M \times [HA]} \qquad (2)$$

where M is the molecular weight of the salt, the value 10 represents Gm./L. of salt for a 1 per cent solution, and % is the concentration of the sodium sulfonamide desired. Letting $\frac{10}{M \times [HA]}$ be equal to $N$, the equation reduces to

$$pH = pK_a + \log (N \times \%) \qquad (3)$$

Table I gives the $N$ values for several sulfonamides as calculated from available data.

The theoretical solubility curves for the sulfonamides may be calculated from Eq. 3 by substituting the proper $N$ values as listed in Table I. The solid black lines in Fig. 1 represent the solubility of sulfathiazole sodium and sulfadiazine sodium as a function of *p*H calculated from Eq. 3. Experiments were devised to test the validity of this equation for solutions of sulfathiazole sodium in which the *p*H was adjusted by means of hydro-

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‡ Assistant Professor of Pharmacy, Temple University.
[1] These data pertain to commercial samples which were supplied by Calco Chemical Division of the American Cyanamid Company.

TABLE I.—SOLUBILITIES AND $N$ VALUES FOR SEVERAL SULFONAMIDES

| Drug | Molecular Weight | Solubility of Acid Form at 25°, Moles/L. | $pK_a$ | $N$ |
|---|---|---|---|---|
| Sulfathiazole sodium | 304.33 | 0.00235[a] | 7.12[b, c] | 14.0 |
| Sulfadiazine sodium | 272.27 | 0.00031[a] | 6.48[b, c] | 118.5 |
| Sulfamerazine sodium | 286.29 | 0.00060[a] | 7.06[c] | 58.1 |
| Sulfapyridine sodium | 289.29 | 0.00113[a] | 8.43[b]<br>8.44[c] | 30.6 |

[a] Calculated from the U. S. P. and N. F. solubilities which are in agreement with the best experimental values.
[b] Bell, P. H., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, **64**, 2905(1942).
[c] Northey, E. H., "The Sulfonamides and Allied Compounds," Reinhold Publishing Corp., New York, 1948, p. 461.

chloric acid, and also for solutions of the sulfonamide salt in borate buffers.

## EXPERIMENTAL

**Unbuffered Solutions.**—Five per cent aqueous solutions of the sodium salts of sulfathiazole, sulfadiazine, sulfamerazine, and sulfapyridine were prepared and the $p$H[2] of these solutions was determined at 25°.

**Solubility of Sulfathiazole Sodium Solutions Adjusted with Hydrochloric Acid.**—Solutions were prepared by dissolving approximately 10 Gm. of sulfathiazole sodium in 50 ml. of distilled water in a 125-ml. glass-stoppered Erlenmeyer flask. The $p$H of the solution was reduced to the desired point by the dropwise addition of hydrochloric acid. The flasks were placed in a constant temperature bath at 25 ± 0.5° and agitated intermittently for forty-eight hours. At the end of this period, the excess solute was permitted to settle and 10 ml. of solution was drawn off with a pipette, the tip of which was fitted with a short length of rubber tubing and a glass tube plugged with cotton to serve as a filter. This solution was transferred to a 250-ml. beaker and assayed according to the official assay for sulfathiazole sodium U. S. P. (2).

A $p$H determination was made on the mixture in the flask immediately following withdrawal of the 10-ml. sample of solution. Duplicate $p$H determinations and assays were carried out on each sample.

**Solubility of Sulfathiazole Sodium in Borate Buffer.**—Clark-Lubs borate buffers, modified by deleting the potassium chloride, were employed. Excess sulfathiazole sodium was added to 50-ml. portions of buffer solutions at various $p$H values. The mixtures were placed in 125-ml. glass-stoppered Erlenmeyer flasks, placed in a constant temperature bath at 25 ± 0.5°, and agitated intermittently for forty-eight hours. At the end of this period a 10-ml. sample of the clear solution was removed and assayed as described previously. A $p$H determination was made on the mixture at the time of the removal of the sample for assay. Duplicate $p$H determinations and assays were carried out on each sample.

While the majority of points obtained were on the theoretical curve calculated from Eq. 3, several values were obtained which indicated the formation of highly supersaturated solutions. It is possible that these results may have been due to hydration of the sulfathiazole in solution and the production of a form which could not crystallize out since there was no solid form of this hydrate present. Northey (3) pointed out this phenomenon in discussing the differences in reported solubilities of sulfamethazine.

**Increase in pH of Buffer Solution on the Addition of Sulfathiazole Sodium.**—The sodium sulfonamides are strongly alkaline and, when added to a buffer of definite $p$H, will raise the $p$H of the system appreciably. Thus, in order to dissolve a sulfonamide of a given concentration, a buffer may be used which has a $p$H below that of the final solution. As shown in Fig. 1, a $p$H of approximately 8.8 is necessary for the dissolution of a 3% concentration of sulfathiazole sodium. The initial $p$H of the buffer need only be 8.7 to obtain this final value.

For this work it was necessary to prepare a sulfathiazole sodium solution which was free from any excess sodium hydroxide. The sodium hydroxide-free salt was prepared by adding 300 Gm. of sulfathiazole and 40 Gm. of sodium hydroxide to 1 L. of distilled water in a 2-L. Erlenmeyer flask. The mixture was stirred, heated to 50° on a water bath, and maintained at this temperature for twenty minutes. The flask and contents were then cooled in an ice bath and filtered while cold in order to minimize the quantity of free sulfathiazole present in the filtrate. The solvent was evaporated and the salt thus obtained was used in all experiments involving an increase in $p$H of the buffer solution.

Since reproducible $p$H values could not be obtained by the simple addition of excess sulfathiazole sodium to the buffer solution, the drug was added in increments until the $p$H ceased to vary by more than 0.02 $p$H units. Table II indicates the initial and final $p$H values of the buffer solution, and the concentration of sulfathiazole sodium which the solution should be capable of holding.

The curve illustrated in Fig. 2 was obtained by plotting the initial $p$H of the buffer solution *vs.* the concentration of sulfathiazole sodium (Fig. 1) which the buffer solution should hold at the final $p$H.

## DISCUSSION

The present investigation has shown that the precipitation of sulfonamides in borate buffers is not due to a chemical incompatibility of sulfonamide and borate, but is simply a function of the $p$H of the solution. Using a modified buffer equation (Eq. 3), it can be shown that a $p$H of 8.97, rather than 8.6 is necessary for complete solubility of sulfathiazole in concentrations of 5%.

[2] $p$H determinations were made with a Beckman $p$H meter employing a type "E" glass electrode for $p$H values above 9.0.

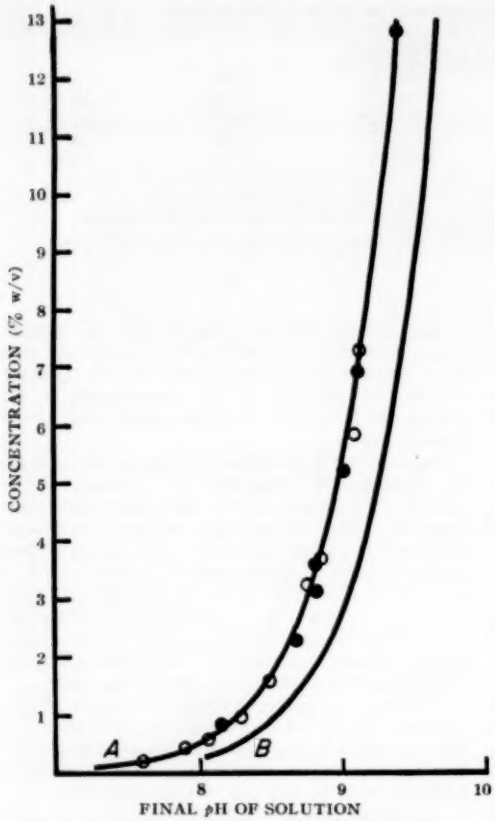


Fig. 1.—Solubility of sodium sulfonamides at 25°. *A*, sulfathiazole sodium; *B*, sulfadiazine sodium. Solid lines indicate theoretical curves calculated from Eq. 3. ○ : experimental values in water. ● : experimental values in borate buffer.

A table of $N$ values has been formulated for the sodium salts of a number of sulfonamides. Substitution of the appropriate $N$ value in Eq. 3 permits calculation of the pH required for the solubilization of any one of these salts.

The fact that sodium sulfonamides, due to their marked alkalinity, will alter the pH of buffer systems has been considered in this investigation. The increase in pH permits the dissolution of additional sulfonamide and thus the initial pH of the buffer may be somewhat lower than that of the final solution desired. Although this has been tacitly assumed in previous investigations, the authors have presented quantitative data to demonstrate the phenomenon. This alteration of pH may be observed by comparing the sulfathiazole curves in Figs. 1 and 2.

**Preservation and Stabilization.**—Solutions of the sodium sulfonamides are subject to mold growth and also undergo a color change on aging, becoming yellow to red-brown. Although Whittet (4) has stated that this discoloration does not increase toxicity nor alter therapeutic activity, it is nonetheless undesirable. Preservation of the solutions

TABLE II.—CHANGE IN pH OF BORATE BUFFER ON ADDITION OF SULFATHIAZOLE SODIUM

| Initial pH of Buffer | Final pH of Solution | Concn.[a] of Sulfathiazole Sodium (% w/v) |
|---|---|---|
| 7.60 | 7.88 | 0.45 |
| 7.80 | 8.06 | 0.60 |
| 7.88 | 8.18 | 0.80 |
| 8.00 | 8.22 | 0.90 |
| 8.26 | 8.41 | 1.40 |
| 8.40 | 8.51 | 1.75 |
| 8.48 | 8.62 | 2.20 |
| 8.58 | 8.65 | 2.40 |
| 8.68 | 8.78 | 3.25 |
| 8.72 | 8.78 | 3.25 |
| 8.80 | 8.93 | 4.65 |
| 8.89 | 9.01 | 5.50 |
| 9.01 | 9.11 | 7.00 |

[a] The concentration of sulfathiazole which the buffer is capable of holding at the final pH, as determined from the curve shown in Fig. 1.

presents a difficulty since many of the commonly used ophthalmic preservatives are not acceptable. Benzalkonium chloride is incompatible with sulfathiazole; phenylmercuric nitrate is incompatible with sulfadiazine; chlorobutanol is not useful at the highly alkaline pH; and the *p*-hydroxybenzoates must be eliminated because of the irritation which they produce.

Exsiccated sodium sulfite in 0.1% concentration effectively retarded the discoloration for a period of four to five weeks. It is recommended that the solutions be prepared with clean equipment, stored in a cool place, and dispensed in dark bottles.

While Fig. 2 indicates that a buffer with an initial pH of 8.90 is suitable for the preparation of a 5% solution of sulfathiazole sodium at 25°, it is desir-

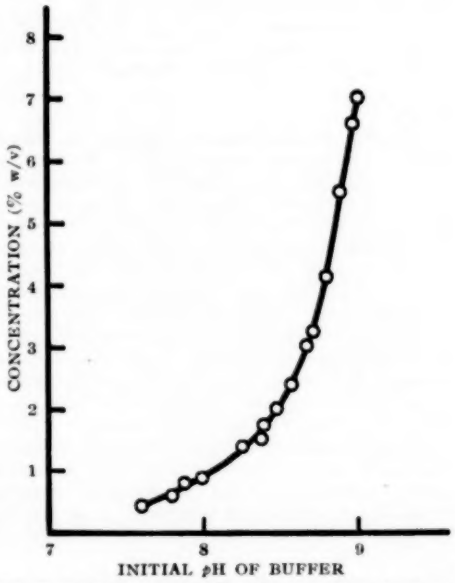


Fig. 2.—Solubility of sulfathiazole sodium in borate buffer at 25°.

able from a practical standpoint to employ a buffer of *p*H 9.00 to diminish the possiblity of precipitation during storage at variable room temperatures. The following formula is recommended as a buffer solution for ophthalmic solutions of sulfathiazole sodium in concentrations of 5%.

| | |
|---|---|
| Boric Acid | 0.43 Gm. |
| Sodium Borate | 4.20 Gm. |
| Sodium Sulfite, Exsiccated | 1.00 Gm. |
| Distilled Water, enough To make | 1,000 cc. |

Borate buffers at somewhat higher *p*H values might be employed for solutions of sulfadiazine sodium and sulfamerazine sodium. However, there is little to be gained by this since the calculated minimum *p*H required for a 5% solution of either of these salts is very near to the *p*H value of a 5% solution in distilled water. This close approximation may be attributed to the extremely slight solubility of the acid form of these two salts. It is therefore believed advisable to prepare sulfadiazine sodium and sulfamerazine sodium solutions in distilled water, with the addition of 0.1% exsiccated sodium sulfite.

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# The Effects of Cortisone and Desoxycorticosterone on the Toxicity of Barbiturates*,†

By CHARLES K. GORBY, CHARLES A. LEONARD, JULIAN L. AMBRUS, and JOSEPH W. E. HARRISSON‡,§

**The effect of cortisone and desoxycorticosterone acetate in barbiturate poisoning was investigated. The toxic effect of low doses of pentobarbital was decreased by cortisone, while that of higher doses was increased by desoxycorticosterone. Both cortical steriods appeared to increase the toxicity of phenobarbital. The mechanism of this phenomenon is discussed.**

WINTER AND FLATAKER (1) have reported that cortisone provided partial protection in mice against toxic doses of methadon and decreased the thermoanalgesic effect of morphine and methadon. These workers also showed that cortisone lengthens the induction time of ether anesthesia and reduces its duration. Desoxycorticosterone acetate (DCA) slightly increases sleeping time and antagonizes the effect of cortisone. Cortisone shortens the sleeping time of Evipal anesthesia, while DCA has no effect (2). Boswell (3) and Fraser, *et al.* (4), reported the usefulness of cortisone in the clinical treatment of barbiturate-withdrawal syndrome. Trethwie and Gladwell (5) presented indirect evidence indicating that an impaired response of the adrenal medulla may follow the administration of barbiturates. These data aroused interest in the effect of cortisone and desoxycorticosterone acetate on the effect of toxic doses of barbiturates.

## MATERIALS AND METHODS

Male Swiss mice, 20–25 Gm., were used. They were maintained on Rockland complete rat checkers and water *ad lib.*, and were housed in air-conditioned quarters at a temperature of 21°.

Cortisone acetate (Cortone Acetate Merck)[1] and desoxycorticosterone acetate (DCA)[2] were injected subcutaneously in a dose of 100 mg./Kg./day for six days. On the sixth day the barbiturates were administered intraperitoneally. Pentobarbital sodium (Nembutal, Abbott Labs.)[3] and phenobarbital sodium, U. S. P., were used, representing a medium- and a long-acting barbiturate, respectively. The percentages of deaths in the different dose level groups were calculated according to the method of Behrens (6).

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† Supported in part by a grant from Eli Lilly and Co.
‡ With the assistance of George V. Rossi.
§ The authors wish to express their indebtedness for the helpful advice and criticism of Dr. Ivor Griffith, Director of Research.

[1] We are greatly indebted to Dr. George Merck of Merck and Co. for supplying us with cortisone.
[2] Our sincere thanks are due to Dr. John N. McDonnell of Schering Co. for providing us with this material.
[3] Part of this material was supplied by the Abbott Laboratories, for which we wish to express our thanks.

TABLE I.—THE EFFECT OF CORTISONE AND DESOXYCORTICOSTERONE ON THE TOXICITY OF PENTOBARBITAL

| Pentobarbital, Mg./Kg. | Control Group | | DCA Group | | Cortisone Group | |
|---|---|---|---|---|---|---|
| | Deaths/N | Behrens, % | Deaths/N | Behrens, % | Deaths/N | Behrens, % |
| 110 | 1/5 | 3.33 | 1/5 | 3.03 | 0/5 | 0 |
| 120 | 2/5 | 10.70 | 2/5 | 9.67 | 0/5 | 0 |
| 130 | 2/3 | 18.50 | 5/15 | 24.20 | 1/15 | 2.56 |
| 135 | ... | ... | 2/7 | 40.00 | 4/9 | 17.20 |
| 140 | 11/21 | 43.50 | 12/18 | 68.80 | 6/18 | 36.00 |
| 150 | 5/15 | 65.50 | 10/14 | 94.00 | 8/15 | 73.00 |
| 155 | 7/8 | 96.50 | ... | ... | ... | ... |
| 160 | 10/10 | 100.00 | 10/10 | 100.00 | 10/10 | 100.00 |

TABLE II.—THE EFFECT OF CORTISONE AND DESOXYCORTICOSTERONE ON THE TOXICITY OF PHENOBARBITAL

| Phenobarbital, Mg./Kg. | Control Group | | DCA Group | | | | Cortisone Group | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pretreated | | Post-treated | | Pretreated | | Post-treated | |
| | Deaths/N | Behrens, % | Deaths/N | Behrens, % | Deaths/N | Behrens, % | Deaths/% | Behrens, % | Deaths/N | Behrens, % |
| 215 | 1/10 | 3.84 | 1/5 | 16.7 | 2/5 | 22.2 | 2/5 | 33.3 | 1/5 | 12.5 |
| 225 | 4/10 | 23.80 | 5/5 | 85.7 | 3/5 | 55.6 | 5/5 | 87.5 | 4/5 | 62.5 |
| 235 | 6/10 | 52.40 | 3/4 | 90.0 | 4/5 | 81.8 | 4/5 | 91.6 | 4/5 | 81.8 |
| 245 | 8/10 | 76.00 | 5/5 | 100 | 4/5 | 92.9 | 5/5 | 100 | 4/5 | 92.9 |
| 255 | 7/10 | 86.50 | 4/4 | 100 | 5/5 | 100 | 4/4 | 100 | 5/5 | 100 |
| 265 | 5/6 | 97.00 | ... | ... | ... | ... | ... | ... | ... | ... |

## RESULTS

Pentobarbital sodium in normal saline solution was administered intraperitoneally in doses ranging from the $LD_0$ to $LD_{100}$ dose. The results are shown in Table I. Pretreatment of animals with cortisone seems to offer a slight protection if the doses of pentobarbital are below the $LD_{50}$. Above this dose, no protection is evidenced. DCA, on the other hand, seems to have little effect if the pentobarbital doses are below the $LD_{50}$, but slightly increases its toxic effect above this dose.

The effect of cortisone and DCA on toxic doses of phenobarbital sodium was tested as described above. A second group was not pretreated with cortisone or DCA, but one dose of 100 mg./Kg. of the respective cortical hormones was injected immediately after the administration of phenobarbital ("post-treated group"). If the animals in coma lived longer than twenty-four hours, a second dose of the hormones was given. The composite results are set forth in Table II. Both cortical steroids seem to increase the toxic effect of phenobarbital.

## DISCUSSION

The reasons for the difference in the effect of cortical hormones on pentobarbital and phenobarbital poisoning are still not known. It must be considered, however, that although the animals died within five hours after pentobarbital administration, after phenobarbital administration they died within approximately thirty hours. It is possible that during prolonged sleep, factors which may be influenced by the high level of cortical hormones become operative. The antagonistic effect of cortisone toward pentobarbital intoxication seems to be slight, since it could be demonstrated only with low dose levels of the latter drug. Winter and Flataker (2) showed the existence of a similar antagonism to hypnotic doses of Evipal. The synergistic effect between desoxycorticosterone and pentobarbital, on the other hand, was manifest only with high doses of the latter.

## SUMMARY

1. Pretreatment with desoxycorticosterone or cortisone appeared to have little effect on pentobarbital intoxication in mice. Cortisone exerted a slight protective effect on doses below the $LD_{50}$ of pentobarbital, while desoxycorticosterone slightly increased the toxicity of doses above the $LD_{50}$ of pentobarbital.

2. Both cortical steroids, whether administered subsequent to the administration of phenobarbital or throughout a six-day pretreatment period, increased the toxic effect of phenobarbital sodium.

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# REFERENCE STANDARDS FOR BIOLOGICAL STANDARDIZATION

In a little more than two decades, biological standardization has evolved from a barely tolerated, dull tool to an accepted and respected method of drug assay. Although it was once regarded as a means of last resort in evaluating products not amenable to other forms of standardization, there are now cases where it is the method of choice. One of the principal reasons for this profound change in status has been the introduction of suitable reference standards which have been made available for every official biological assay of drugs and vitamins.

The practice of using the standards to reduce the variability inherent in a biological system is so firmly fixed that all too often the standards themselves are taken for granted. This is unfortunate only in the fact that those who owe a debt of gratitude for this service may fail to appreciate the enormous labor of selecting, testing, and distributing the standards.

In the United States, five agencies play a role in one way or another in providing reference standards. These are the AMERICAN PHARMACEUTICAL ASSOCIATION, the Bureau of Animal Industry, the Food and Drug Administration, the National Institutes of Health, and the United States Pharmacopeia. On April 7, 1952, the three organizations most concerned participated in a symposium held at the Pharmacopeia Building in New York City. This meeting also provided an unusual opportunity for a review of the broad WHO program on international standards, which was presented most ably by Dr. A. A. Miles, former Chairman of the WHO Expert Committee on Biological Standardization and now head of the Lister Institute, London, Eng.

A possible source of regret is that limitations on time made it unfeasible to cover in the symposium the numerous reference standards which are used in chemical and physical assays. These would include certain U. S. Pharmacopeia Reference Standards, National Formulary Reference Standards, and a large number of analyzed composition-of-matter standards issued by the National Bureau of Standards. However, although the latter number in the hundreds, only a few are of interest to the pharmaceutical chemist.

Without doubt, readers of THIS JOURNAL will welcome the opportunity to read of the service rendered by the organizations which make available these indispensable aids to biological standardization.

# U. S. P. Reference Standards for Biological Assay*

By ADLEY B. NICHOLS†

BIOLOGICAL ASSAYS were given their first Pharmacopeial recognition in U. S. P. IX which appeared in 1916. While only the Cannabis assay was obligatory, procedures were also included for Aconite, Digitalis, Strophanthus, Squill, and Dried Suprarenals. It should be noted, however, that while a biological standardization was required for Solution of Hypophysis, now known as Posterior Pituitary Injection, no method was described except for a reference to the procedure of the United States Hygienic Laboratory of the Public Health Service. This method used histamine hydrochloride as the reference standard in a comparison on the isolated uterus of the virgin guinea pig.

The Aconite assay required no reference standard; its potency was determined on the basis of the minimum lethal dose when administered to guinea pigs.

Cannabis was assayed against a standard fluidextract by producing incoordination in dogs. The standard was prepared by the operator.

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† Executive Assistant to the Director of Pharmacopeial Revision.

Digitalis was assayed by the one-hour frog method. In this case, however, the minimum lethal dose was compared to that of crystalline ouabain, since it was recognized that frogs differed in their susceptibility to these two drugs at different seasons of the year. However, no specific lot of ouabain was designated as a standard because the purity specifications were given in the section on U. S. P. Reagents.

In the case of Dried Suprarenals, the assay was based upon the blood pressure of dogs, with epinephrine of reagent purity used as a standard.

These optional U. S. P. IX biological assay methods were included to encourage manufacturers to study the provision of a means of achieving uniformity in conforming to the proposed standards. All preparations thus assayed and standardized could be labeled "Physiologically assayed and standardized according to U. S. P. IX."

When U. S. P. X appeared in 1926, biological assay methods became mandatory for the abovementioned preparations and also for Aconite and Ergot. At the same time, an optional biological assay was provided for vitamin A in Cod Liver Oil but no reference standard was specified. The U. S. P. X preparations of Cannabis and Ergot, however, required a comparison with standard fluidextracts prepared from a composite of ten different lots of the respective drug. Similarly, Solution of Pituitary was assayed against a standard pituitary powder prepared from not less than 25 fresh posterior lobes of the pituitary body of cattle, and Solution of Epinephrine Hydrochloride was assayed against a standard epinephrine hydrochloride prepared from reagent epinephrine as directed.

## FIRST U. S. P. REFERENCE STANDARD

These new requirements in U. S. P. X led to the establishment of the first specific Reference Standards for U. S. P. use. The Bureau of Chemistry of the U. S. Department of Agriculture, forerunner to the present Food and Drug Administration, offered to make available the standard preparations described by the Pharmacopeia. Under this agreement the Bureau distributed epinephrine, ouabain, posterior pituitary, and fluidextracts of cannabis and ergot for almost a decade until the Pharmacopeia undertook this responsibility. This service was a tremendous aid to the pharmaceutical industry as it gave additional assurance of uniformity through the use of common standards by the entire industry.

Early in 1932 the Pharmacopeia issued an interim revision announcement modifying the assay procedure for Fluidextract of Ergot and requiring that the standard fluidextract be aged for three months before being standardized to match the potency of the previous fluidextract distributed by the F. D. A. Further changes were necessitated later when it was discovered that the "standard" was undergoing marked deterioration. This unfortunate situation prompted the F. D. A. to notify the Pharmacopeia that it must withdraw from further distribution of standards when current supplies were exhausted.

As a result the Pharmacopeia underwent another interim revision on January 1, 1935, in which the standard fluidextract was replaced with Ergotoxine Ethanesulfonate as the Reference Standard for ergot preparations. This standard, purchased in sealed tubes containing 12 mg. each and distributed directly from the U. S. P. office, was the second official U. S. P. Reference Standard (U. S. P. Cod Liver Oil Reference Standard, discussed below, was the first Standard.) The biological assays for Ergot and its fluidextract were retained in U. S. P. XI, but because of the instability and waning therapeutic value of the fluidextract, it was included without a potency standard or assay in U. S. P. XII. This action resulted in the deletion of both ergot and its fluidextract from U. S. P. XIII. Hence, while ergotoxine ethanesulfonate was no longer necessary for biologic assays, it was retained for the colorimetric assays of the pure ergot alkaloids which then became official. In 1950 it was replaced with ergonovine maleate which now serves as the Reference Standard for the salts of both ergotamine and ergonovine.

With the appearance of U. S. P. XI, official U. S. P. Reference Standards now required and available were Aconitine, Cod Liver Oil (standardized both for its A and its D content), Digitalis, Epinephrine, Ouabain, and Ergotoxine Ethanesulfonate. The assay for Posterior Pituitary still required the preparation of a standard powder to be prepared by the assayist. However, the Second Bound Supplement to U. S. P. XI called for an official Posterior Pituitary Reference Standard.

The U. S. P. XII saw the addition of several new biological assay standards and many more were added in U. S. P. XIV and XV. There are now 22 such Standards or related substances available.

## THE U. S. P. REFERENCE STANDARDS COMMITTEE

U. S. P. Reference Standards were first prepared with the advice and help of the committees or advisory boards responsible for the official preparations requiring the Standards. About 1945, it was suggested that the status of official Reference Standards would be considerably strengthened if their preparation was more closely supervised by the Revision Committee. Accordingly, the U. S. P. Reference Standards Committee was formed and has continued. It now consists of the Chairmen of the three U. S. P. subcommittees concerned with chemical problems, the Chairman of the Subcommittee on Biological Assays, and the Director of Revision. Proposals for new Reference Standards are still initiated by the individual committees or advisory panels and then submitted to the Reference Standards Committee. Final reports are carefully analyzed and have the approval of the Committee before the Standard is released.

## POLICIES

Most Pharmacopeial Reference Standards have been adopted because they were required for official U. S. P. assays. A few were introduced to aid in developing pharmacopeial assay procedures. These were usually for preparations which had received the approval of the U. S. P. Subcommittee on Scope but monographs for which were incomplete because adequate assays were lacking. Notable examples in this category were estrone, estradiol, estradiol benzoate, progesterone, and testosterone propionate. In the meantime, the release of these Standards led to their extensive use in unofficial assay procedures. It is interesting to note that later, just as a biological assay for estrone was perfected and about to be included in U. S. P. XIV, a chemical assay became available and was adopted. However, the Standard is required in the test for equilin and equilenin.

This policy had been established by the U. S. P. Board of Trustees and was broadened only recently. Because the Board had observed the confused state of affairs which often developed in a new therapeutic field when no common basis existed for the standardization of preparations, it reviewed the entire U. S. P. program and authorized the preparation of Standards where they were expected to advance the practice of medicine and pharmacy and assist research workers in general, even though there was no immediate or contemplated need for them in the U. S. P.

A fixed policy governs the selection of starting material to be used in preparing a Reference Standard. Where a Standard represents a pure crystalline substance it matters little whether it is obtained from one source or from several. All should be of equal purity. However, for Standards belonging to the heterogeneous group, potencies and degrees of purity vary considerably. Consequently, for this class it is the policy to obtain material from several sources, which helps to eliminate obvious difficulties and subsequent repercussions.

Another principle is that of purchasing the basic substance required for preparing a Reference Standard. Manufacturers often graciously offer it as a gift but many times its intrinsic value is very high. In such cases it would be manifestly unfair for one firm to bear virtually the entire cost for the industry. When this occasionally happened in former days, the U. S. P. distributed the Reference Standard without charge, until it was found that unfair advantage was taken of this practice. When required materials are purchased, a nominal charge for the Standard can be made, depending upon the approximate costs. This charge covers most of the cost of conducting the Reference Standards program; however, part of the expense is still borne by the U. S. P. in keeping with the attitude of the Board of Trustees that a basic service is rendered by making these Standards available.

If an International Standard is available, it is the policy to prepare the U. S. P. Reference Standards in conformity with it. Where possible, a joint program is conducted in such a manner that the resulting Standard can be shared. The U. S. P. Standards and the International Standards are identical in these cases.

## U. S. P. AND INTERNATIONAL UNITS

A policy established so firmly that it scarcely need be mentioned is the identity of the U. S. P. and International Units of activity. Although there is not a U. S. P. Unit corresponding to every International Unit or vice versa, wherever the two coexist they are equal in magnitude. From this one might question the need for U. S. P. Units. However, for the purposes of the F. D. A. enforcement program it has been considered necessary to date to establish U. S. P. Units whenever they are needed in connection with a U. S. P. monograph. The real necessity for this practice might be argued from a scientific standpoint, but it is probably an issue to be resolved by legal experts. That is, from a scientific and pharmacopeial viewpoint, standardization of U. S. P. products in International Units is desirable.

## TABLET AND CAPSULE FORMS

One of the few advantages of biological assay over chemical assay is an occasional instance of much greater sensitivity. The microbial assay of cyano cobalamin is a good example. With this assay, using *Lactobacillus leichmannii*, concentrations as low as 0.00002 μg. per cc. are readily assayed, compared with 5 μg. per cc. required for the chemical method which itself represents remarkable sensitivity for a colorimetric procedure. The necessary Reference Standards for biologic procedures often represent rare and costly substances and some degree of conservation must be maintained. The combination of these factors results in the desire to measure minute quantities of the Standards, often smaller than can be handled accurately even on a micro balance.

Occasionally the active principle of the Reference Standard is somewhat unstable in crystalline form, e. g., vitamin A acetate and vitamin $D_3$. Forms of Standards which are stable for indefinite periods are thus needed.

These various needs are met by using, when necessary, soluble tablets for potent, stable Reference Standards, as in the case of Heparin Sodium, and soft gelatin capsules of oil solutions of the unstable oil-soluble, crystalline Standards.

The quantities contained in each capsule or tablet are estimated to be sufficient for only a short period, varying from a day to about a week. In this manner, a stable standard preparation is obtained quickly and conveniently.

These forms of Standards have met with enthusiastic approval, and analysts generally have found them accurate and convenient. They eliminate a real source of potential error in carrying out the official assays which are needed to maintain the high standards almost universally characteristic of American pharmaceuticals.

The weight of individual tablets varies enough to necessitate weighing each tablet before use Labeled potencies are expressed in terms of potency or activity per mg. of tablet, and although tests have proved that the tablets are homogeneous, no less than one tablet should be used at a time for the sake of accuracy.

U. S. P. Vitamin A and Vitamin D Reference Standards are supplied in capsule form. Corti-

cotropin, Heparin Sodium, Posterior Pituitary, and Thyrotropin are supplied as tablets.

## STORAGE OF THE U. S. P. REFERENCE STANDARDS

Every effort is made to store the packaged and bulk stocks of the U. S. P. Reference Standards under conditions favorable to maximum stability. Most of the Reference Standards require only a dry atmosphere and protection from excess heat and exposure to air. Control of moisture and its effects is difficult, and through experience it has been found necessary to install special dehumidifying equipment to safeguard the Standards against damage from high humidity.

Some of the Standards, such as insulin and powdered digitalis, are sufficiently sensitive to air and moisture to require packaging in glass containers sealed by fusion. The glass for these containers is selected with care to reduce to a minimum the risk of contaminating the contents with glass fragments in opening the vials.

## HETEROGENEOUS VS. CRYSTALLINE REFERENCE STANDARDS

U. S. P. biological Reference Standards may be divided into two general groups. One group includes those which are available in purified or crystalline form, and which usually offer no serious difficulties in preparation. It will be seen later, however, that many difficulties may arise when such a substance is used in minute quantities. The other group represents natural or heterogeneous products, the potency of which varies from lot to lot and from which the active ingredients have not been isolated satisfactorily. Clearly defined characteristics such as melting point, specific rotation, and simple gravimetric or volumetric assays are lacking for this group.

**Digitalis.**—U. S. P. Digitalis Reference Standard is a striking example of the tremendous amount of time, effort, and expense involved in preparing a heterogeneous U. S. P. Reference Standard.

It was pointed out earlier that ouabain was first used on frogs as a Reference Standard for digitalis and related cardiac drugs. Its action was representative of this group of preparations, and because of its crystalline nature, its identity as a specific entity was simple. However, ouabain proved to be unsatisfactory, particularly from an International standpoint, because frogs varied in their response to ouabain; also, this response was not always identical with that obtained from the drug assayed. Accordingly, in 1926 an International Standard of powdered digitalis representing a blend of 10 samples of digitalis was developed. This led to the adoption of an International Digitalis Unit represented by the activity of 0.1 Gm. of the International Standard.

As time elapsed, it was evident that the U. S. P. X digitalis assay, using the one-hour frog method with ouabain as the Reference Standard was not entirely satisfactory. More and more evidence appeared of marked variability in the results obtained with this assay by different laboratories. Much attention was given to extending the one-hour method to four, twelve, and eighteen hours, respectively, and the use of a cat method of assay was advocated. Manufacturers used various methods and physicians were greatly confused in attempting to correlate units of one method with those of another. Actually, there was no direct relationship among the various methods. When a group of manufacturers conducted a collaborative study on some digitalis tinctures and found variations of 80–200% in reported potencies for the same tincture, the critical nature of the situation was clearly recognized.

It was agreed that the one-hour frog method should be continued for U. S. P. XI but ouabain should be replaced with a standard digitalis powder as a Reference Standard. This powder was to be standardized against the International Standard and a U. S. P. Unit identical with that of the International was to be adopted.

This led to the establishment of the first U. S. P. Digitalis Reference Standard, a blend of three lots of high quality digitalis, two-thirds from an American source, one-sixth from England, and one-sixth from Germany. The composite powder was assayed under the direction of Dr. C. W. Edmunds of Ann Arbor, Mich., who was at that time chairman of the U. S. P. Subcommittee on Biological Assays. Several methods were used, all of which showed a high potency for the new Standard. It was thus concluded that 0.745 Gm. of the powder was equivalent in potency to 1 Gm. of the International Standard.

While the adoption of a U. S. P. Digitalis Reference Standard aided the situation to some extent, it did not solve all the problems which had been under discussion. In the ensuing years, many articles appeared attacking the official assay method and the Reference Standard. Some of these were evidently based on misunderstanding or improper use of the official assay.

The adoption of a new Reference Standard based upon the International Standard and Unit had been expected to increase the potency of U. S. P. XI digitalis 25–30% above that of U. S. P. X digitalis. This information was widely distributed to physicians to assist them in the prescribing of these preparations. Actually, subsequent events proved that the increase was greater than had been anticipated and the factor 0.745 should have been about 0.67. There were other developments such as the introduction of biometric principles in the assay itself, which led to another collaborative study in 1939.

The great value, however, of collaborative effort in arriving at a new lot of Reference Standard and a new method of assay was illustrated in the 1939–1942 digitalis assay study in which more than 60,000 frogs were used by the 10 collaborating laboratories and 616 cats were used by 16 laboratories. Initial plans for this study were formulated at Atlanta, Ga., in 1939. The study had a twofold purpose, *viz.*, to develop a more satisfactory method of assay for U. S. P. XII and to provide a new lot of Reference Standard to replace that of 1936 which was nearly depleted. This principle of simultaneously developing or improving an assay method and standardizing a Reference Standard yields rich benefits not only by providing extensive data on the potency of the Standard by the official assay method but also by insuring general familiarity

with its use. The entire program was supervised by a steering committee composed of experts actively engaged in assay work of this type.

Four lots of digitalis leaves were obtained and a preliminary assay was made on each to determine its suitability for the study. These four lots plus 20 lb. of the original 1936 Reference Standard, a total of 110 lb., were ground to uniform fineness, thoroughly mixed, and carefully dried.

The first assay on frogs was made on a separate lot of digitalis of high potency, together with a dilution of the digitalis, as a preliminary determination of the relative values of the one-hour and the eighteen-hour methods. From this assay it was concluded that the eighteen-hour frog method was the most accurate. A second comparison involved hot and cold extraction of the composite powder, using a tincture as the common base of reference. The cold extraction procedure was shown to be superior.

A third comparison served to standardize the composite study powder against the 1936 International Standard by both the one-hour and the eighteen-hour frog methods. Again the eighteen-hour procedure proved to be superior.

The fourth comparison involved an assay on a trial dilution of the U. S. P. composite powder to 1 U. S. P. (and International) Unit per 0.1 Gm., using exhausted digitalis marc. Because of the results of this assay, approval was now given to dilute all of the study powder to the desired level.

The decision to eliminate a "factor" by having the Reference Standard adjusted exactly to the potency of the official drug reflected the unfortunate experience and confusion associated with the first U. S. P. Digitalis Reference Standard.

The fifth comparison, conducted in August, 1941, was made on the supposedly final dilution of the composite powder. This provided the final standardization against the 1936 International Standard Digitalis and gave desired information on a two-dose design of assay (using frogs) together with data on the uniformity of standard tincture prepared from a standard powder.

The data of these study comparisons were critically examined from a statistical standpoint. Each new comparison was based on facts which had been obtained from the previous step. The entire program emphasized the value and the need for carefully planned studies of this nature.

In the meantime, at the end of April, 1941, Dr. Harry Gold first requested a group of associates to cooperate in an assay of digitalis, using cats. The results of this study on two tinctures were so satisfactory that a conference was called in July and plans were made for a further study, in which two previously prepared tinctures were compared on cats with a tincture prepared by each collaborator from the same composite powder used in the study on frogs. At the same time an open conference was scheduled for early in September to review the entire digitalis situation. It must be recalled that U. S. P. XII was then in galley proof and the need for settling how digitalis should be assayed was very urgent.

At the open conference held on September 6, general approval was given to the cat assay method, and this action was subsequently endorsed by the U. S. P. Revision Committee.

A third assay conducted on cats, comparing the already-diluted standard powder with the International Standard, led to a slight adjustment of the powder to make the final Reference Standard which became known as the U. S. P. Digitalis Reference Standard of 1942.

In 1946 it was necessary to replenish the stock of standard dilution of this material in order to provide additional Reference Standard. The remainder of the original basic digitalis powder of 1939 was diluted on the same basis as the 1942 Reference Standard and assays showed a potency of about 104%. When all factors had been considered, it was decided that this dilution should be accepted as identical with the original 1942 Standard. It is still being distributed at this time.

**Corticotropin (ACTH).**—The Pharmacopeia recognizes the difficulty of attempting to arrive at a satisfactory Unit where little is known about the basic substance. This is being forcefully brought to mind in the case of ACTH, or corticotropin.

Several years ago the Pharmacopeia was requested to prepare and distribute as a Reference Standard what was then known as the La-1-A material which was distributed widely by Armour Laboratories for laboratory research. The policy of the U. S. P. in force at that time did not permit this course of action.

Since, then, the Armour standard has become the first International Standard ACTH, a fact which is generally known.

In July, 1951, the Pharmacopeia made available a Corticotropin Reference Standard on relatively short notice to combat growing confusion on the part of physicians and others. The critical nature of this specific problem became apparent with the introduction of commercial ACTH products under effective F. D. A. new drug applications. The F. D. A. required that, in the absence of a practical, common reference standard, each firm should set up its own unit of activity and "house standard". The risk of impending confusion was extremely great.

An offer of a generous supply of purified hormone similar to the original Armour La-1-A and sufficient for a U. S. P. Reference Standard had to be rejected as contrary to established U. S. P. policy.

Representatives of the three firms which had effective new drug applications for ACTH conferred with representatives of the U. S. P., A. M. A. and F. D. A. in Washington in March, 1951. It was agreed that, with the cooperation of those present, the U. S. P. Reference Standard could promptly be made available and a plan which was devised was quickly approved by the U. S. P. Reference Standards Committee. This plan included the appointment of an Advisory Study Panel of five experts on the assay of corticotropin.

Several grams of ACTH powder were obtained from each of the three firms involved. The entire quantity was thoroughly mixed and diluted with lactose so that each 20 mg. represented 100 μg. of the ACTH powder. This diluted powder was then pressed into 20-mg. tablets without the use of binder or lubricant. The result was the provisional U. S. P. Corticotropin Reference Standard.

The decision to form tablets of the diluted powder was based on the small quantity of hormone needed and the very limited supply. This latter con-

sideration applied particularly to the International Standard.

The contents of several tubes of the International Standard ACTH (La-1-A) were weighed and very carefully diluted with lactose to the same basis as the U. S. P. Standard. The mixture was thoroughly milled to assure uniform distribution of the active substance with the inert diluent. This was very important because these tablets were to be used as the basis for standardizing the U. S. P. tablets and there was apparently no simple way to check the potency of the International tablets themselves.

Five laboratories assayed the U. S. P. tablets against the International, using both the original Sayers method and the Munson modification. On the basis of the results, the Study Panel adopted a factor of 0.120 U. S. P. Corticotropin units per 20 mg. of the Provisional Standard (i. e., 0.006 U. S. P. Unit per mg.). The new Reference Standard was released in July, 1951, with the understanding that further assay data would be obtained and some revision of the potency factor might be necessary in the light of new data. By definition, the U. S. P. Unit was identical with the International Unit which was equal to 1 mg. of the original La-1-A standard.

The program up to this point had taken about four months, a record for a U. S. P. Reference Standard. However, the problem was not settled, for within a few weeks evidence was presented that the International Standard might have lost potency during the preparation of the tablets. The Armour Company had run several assays on the U. S. P.-prepared International tablets against their small reserve supply of original La-1-A and found them noticeably less potent than expected from their content of International Standard. On the assumption that this was caused by the milling operation, a sample of corticotropin similar to La-1-A was milled. Assays showed a significant loss in potency.

With this information, the Study Panel immediately instituted a new study program. The small amount of International ACTH Standard which was available, totaling about 15 mg., was given to a microanalytical laboratory to be weighed in portions of approximately 200 μg.; the recorded weight was considered accurate to 5 μg. These portions were sealed in containers suitable for making up the standard solution for the assay. The results of the 36 assays reported were critically analyzed by statistical methods and led the Study Panel to reduce the potency factor to 0.005 U. S. P. unit per mg.

Because the only assay methods available for ACTH at the present time are not sufficiently accurate due to variations not only among different laboratories but also within individual laboratories, it was not expected that the change in potency of the Reference Standard would have any material effect from a clinical standpoint. Fortunately, however, the adjustment increased rather than decreased the potency of the market products.

**Globin Hydrochloride.**—When globin zinc insulin was admitted to U. S. P. XIV, it was necessary to provide a Globin Reference Standard for the test for Biological Reaction, a measure of the prolongation of the hypoglycemic effect.

A portion of a lot of globin hydrochloride which had been used under similar conditions for some time was made available to the Pharmacopeia. It was derived from beef hemoglobin isolated with the aid of toluene in the presence of suspended aluminum hydroxide and split into the component globin and heme by hydrochloric acid. The resultant globin hydrochloride was dried with acetone and ether and meets specifications based upon its chloride and nitrogen content, moisture, and residue on ignition. It has no biological activity *per se*.

**Heparin Sodium.**—The U. S. P. Heparin Sodium Reference Standard is another example of the heterogeneous group which exemplifies the many unexpected difficulties in preparing a Standard.

The drug heparin sodium was approved for admission to U. S. P. XIII and plans were made in August, 1945, for a suitable monograph and a Reference Standard which was expected to be required for the assay. However, the problems were so involved that only a start was made during the period of active revision for U. S. P. XIII.

Finally in March, 1947, an elaborate collaborative study to furnish information for the development of both a monograph and a Reference Standard was begun. It was proposed that the sodium salt of heparin should be made the official standard and that it should be based upon the Provisional International Heparin Standard which had recently been adopted. Material was obtained from several sources, mixed, purified by precipitation as the barium salt, and finally converted to the sodium salt. However, the resulting product was pyrogenic. While the presence of pyrogens was not expected to interfere with the intended use of this material, it was undesirable to distribute a Reference Standard which did not meet the U. S. P. specifications for heparin sodium.

This necessitated further purification with attendant loss and replacement of active material. The final product was satisfactory in appearance odor, solubility, color of solution, *p*H, protein, barium, moisture, ash, sulfur, nitrogen, safety, nonspecific depressor substances, potency, and pyrogens.

All of the major manufacturers of heparin sodium joined in the collaborative study program. Detailed directions were given for four different methods of assay then in common use and each laboratory applied each method to six samples. The methods included the provisional International Standard; the proposed U. S. P. Standard; a high, a low, and a medium powder; and a high potency solution.

The results of these assays showed good uniformity between the various laboratories, and one of the methods was selected for U. S. P. XIV. The proposed Standard had a rated average potency of 101.4 U. S. P. Units per mg. It was agreed to round off this value to 100 Units. Authorization was given to prepare tablets of the Standard, each containing 50 U. S. P. Units of activity. The tablets were assayed by seven laboratories. It was found that the proposed potency of 50 Units per tablet was not quite achieved, as the weighted potency for all laboratories amounted to 47.6 Units for each 20 mg. of tablet, corresponding to 2.38 Units per mg.

As in other cases where an International Unit exists, the Pharmacopeial Unit has been made identical to the International by definition. The

International Standard Heparin has an activity of 130 I. U. per mg. It is believed that the unit closely approximates that proposed by Murray and Best in 1938, sometimes referred to as the Toronto Unit, which is equivalent to 0.01 mg. of a crystalline barium salt of heparin. The several lots of heparin sodium first mixed together for the U. S. P. had approximately the same potency as the International Standard. However, as successive purifications were made, the relative potency of the material decreased.

**Hyaluronidase.**—If the current policy on U. S. P. Reference Standards had been definitely established several years ago, at present there would be only a single unit, and possibly only a single assay procedure, for preparations of hyaluronidase. In fairness, it must be stated that when work first began on the enzyme, those interested requested the Pharmacopeia to establish a Reference Standard. At that time, there was no immediate prospect of recognizing hyaluronidase in the Pharmacopeia and the request was not approved. Now, however, the general policy has changed and hyaluronidase has been recommended for admission to U. S. P. XV. The Pharmacopeia thus must establish a Reference Standard and a single unit in a field where two different units and separate house standards as well as two methods of assay are firmly established.

A program is under way, however, to set up a Reference Standard and a U. S. P. Unit and to agree on a common assay procedure.

**Posterior Pituitary.**—A biological assay for pituitary first became obligatory in U. S. P. IX, 1916, but it was not until 1936 that an official U. S. P. Reference Standard was available. No assay details were included in U. S. P. IX, but U. S. P. X provided directions for making a standard powder from fresh pituitary. With the appearance of U. S. P. XI, the Pharmacopeia prepared the first lot of U. S. P. Standard. Two replacement lots had been prepared by 1949. All three of these Standards were simply the crude acetone-dried tissue of the posterior pituitary gland. The active hormone had to be extracted by trituration with 0.25% acetic acid, a tedious and uncertain process.

At about this time, however, it seemed possible to lyophilize an acetic acid extract of pituitary and to prepare from this extract a Reference Standard in the form of a soluble tablet. To test this theory, a small amount of lyophilized extract was obtained, diluted with lactose, and made into tablets which were studied for a full year by the A. PH. A. Subcommittee on Physiologic Testing. It was reported that the new tablets, which supplied enough material for single assays, had many advantages over the old Reference Standard powder.

As a result, a new lot of Reference Standard was made up in tablet form. While the tablets were prepared to contain 10 U. S. P. Units per 20 mg., the results of assays conducted by seven laboratories led to acceptance of a potency factor of 9.4 U. per 20 mg. of tablet (0.47 U./mg.).

The record of the numerous comparisons of each new Standard for posterior pituitary with the last and usually also with the prevailing International Standard is extensive and causes uncertainty due to the accumulated errors of biologic assay. Every effort has been made to insure that the U. S. P. Unit, reflected by the potency factor of the Reference Standard, would be unvarying and equal to the International Unit. Recently this factor has been questioned and a carefully planned collaborative assay may be needed to settle all doubts on the potency of the U. S. P. Posterior Pituitary Reference Standard.

**Protamine.**—Protamine was added to the list of U. S. P. Reference Standards when protamine zinc-insulin was recognized in U. S. P. XIII. The protamine, obtained from mature testes of salmon, is a white powder soluble in disodium phosphate solution. It is characterized by its nitrogen and sulfate content.

The protamine itself has no specific biologic activity but is tested to show whether it combines with the U. S. P. Zinc-insulin Crystals Reference Standard to form a prolonged hypoglycemia. This is determined by two procedures, an eleven-hour and a six-hour test on rabbits in which the blood-sugar level is determined hourly and must show a characteristic pattern.

**Thyrotropin.**—Because of the experience with corticotropin and hyaluronidase, the Pharmacopeia accepted a request about a year ago to assist in making available a common Reference Substance for thyrotropin.

Clinical and laboratory research on this hormone had increased. The experimental work was carried out with preparations made available by at least two manufacturers. There was, however, no accepted reference standard which would provide a uniform basis for reporting the results of assays and for labeling products for clinical study.

Because of the lack of adequate information, the Pharmacopeia had to accept a preparation which appeared to be representative and to establish a temporary U. S. P. Unit. Since the material is heterogeneous, the unit basis is the only satisfactory way to express potency. The U. S. P. Unit is defined as the potency of 20 mg. of tablet. Each milligram is therefore equal to 0.05 U. S. P. Unit. This material is now being distributed, and it is hoped that as work progresses it will be found that a service has been rendered in making a common substance available and providing a single unit for expressing its potency.

**Tyrothricin.**—Tyrothricin Reference Standard was added to the list of U. S. P. XIV Standards for use in the microbiological assay of Tyrothricin. Since Tyrothricin consists of 80% of tyrocidin and 20% of gramicidin, separate lots of these two substances were specially purified and checked for suitability before being mixed to prepare the final Standard. Samples of the two basic substances and the mixtures were also tested biologically against an available house standard by the U. S. P. XIV assay procedure. The results showed the expected activities for gramicidin and tyrocidin, respectively, and the mixture, which was made on the same basis as Tyrothricin, showed the expected 100% activity.

**Vitamin A.**—The story of the U. S. P. Reference Standards for Vitamins A and D may be associated with both the heterogeneous and the crystalline groups, inasmuch as the first Standard was Cod Liver Oil while current standards are made from crystalline material. A U. S. P. Reference Standard for vitamin A is the oldest of the U. S. P. Standards and is also the one in most demand. It

is used in the assay of numerous U. S. P. preparations containing vitamin A, ranging from fish liver oils to the highly potent synthetic products of modern pharmaceutical industry.

The first Cod Liver Oil Standard was prepared from four lots of carefully selected destearinated oil which was packaged under $CO_2$ and stored at about 0°. The operation was started in about 1931 but the Standard was not released until the fall of 1933. Sixteen laboratories took part in the intervening study. The Standard became official in May, 1934. The potency of the oil was checked periodically against the International Standard but clear evidence of a change in potency was never obtained.

Late in 1939 a new lot of oil was obtained, which was later known as Cod Liver Oil Reference Standard No. 2. This was treated similarly to oil No. 1 and 14 laboratories took part in its collaborative assay. The physical properties of the oil were also determined in a separate study.

For a number of years, laboratories had been using spectrophotometric analysis, and reports were received that the potency of the Reference Standard varied from bottle to bottle. As a result, the bulk stock of reference oil was reassayed late in 1944 and was immediately packaged as Cod Liver Oil Reference Standard No. 3.

With the development of pure crystalline vitamin A, especially in the form of its relatively stable esters, it was apparent that it was more suitable as a Vitamin A Standard than cod-liver oil. Its relative stability, its ready reproducibility, and its suitability as a standard for the biological assay of vitamin A and also for use in the spectrophotometric method insured broad application and acceptability.

Accordingly a quantity of vitamin A acetate was prepared and examined by four laboratories for its physical and chemical characteristics. Finally, an oil solution was prepared and encapsulated in gelatin to which a light filter had been added. The entire operation was carried out under a blanket of $CO_2$. Thirteen laboratories joined in a final spectrophotometric analysis of the capsules, which were also assayed biologically against both the U. S. P. Cod Liver Oil Reference Standard and the International Carotene Standard. On the basis of the results, the U. S. P. Vitamin Advisory Board assigned a value of 2,500 U. S. P. Units to each capsule. The new Standard was released in July, 1947, and became official on January 1, 1948.

Since the quantity of the new Standard was limited, steps were taken at once to prepare an adequate supply. The same general procedure was followed. The final capsules were shown to be identical in potency with the original Standard and were approved for release in May, 1948.

In scarcely more than a year, the need for a third lot was evident, particularly since a portion of the second lot was set aside for use as an International Standard.

The third lot of vitamin A acetate, used for the Standard in current distribution, was prepared by molecular distillation and thrice recrystallized at −20°. Its specific absorbancy ($E^{1\%}_{1cm.}$) at 325 mμ was 1,535 in ethyl alcohol.

Great care was taken in selecting the diluent oil which was received directly from the processor and examined for peroxide value, tocopherol content, and absorption. Hydroquinone was added to the gelatin capsule mass as an antioxidant and blankets of carbon dioxide or nitrogen were used throughout the entire operation to exclude air.

The oil solution was prepared to contain 3.44 mg. of the vitamin A acetate in each gram. Thus each capsule, which contains 250 mg. of the oil solution, represents 0.75 mg. of vitamin A alcohol.

The capsules were examined by six laboratories and the $E^{1\%}_{1cm.}$ average value of the whole oil at 325 mμ was found to be 5.37.

Statistical analysis of the capsule weights revealed that the accuracy of fill is such that more than 99% of the capsules fall within the limits of 1% of the stated weight of 250 mg. The average weight is 0.2505 Gm.

**Vitamin D.**—The history of the U. S. P. Reference Standard for vitamin D parallels that of vitamin A in the fact that the Cod Liver Oil Reference Standards Nos. 1 and 2 were used for both vitamin A and vitamin D potencies. The Reference Standard is employed for the official assays, using rachitic rats. The potency of numerous U. S. P. preparations is determined by this procedure.

Until recently the International Standard for vitamin D was a solution of irradiated ergosterol. It was recognized that while vitamins $D_2$ and $D_3$ are, weight for weight, equally effective in the rat and in the human being, vitamin $D_2$, also known as calciferol (the form of vitamin D in activated ergosterol), is relatively ineffective for the chick and therefore cannot be used in the determination of the vitamin D content of poultry feed.

The U. S. P. followed the lead of the World Health Organization and also changed to vitamin $D_3$. The new Standard was released November 1, 1950, and became official on May 1, 1951. Several lots of crystalline vitamin $D_3$ were pooled and recrystallized. Crystalline vitamin $D_3$ is not very stable and its physical properties can be determined accurately only on freshly crystallized material prepared on a fairly large scale.

The new Standard is a solution of the recrystallized vitamin $D_3$ in cottonseed oil. Each gram of solution represents 400 U. S. P. Units. The Unit is the same as the International Unit, *viz.*, the biological activity of 0.025 μg. of pure vitamin $D_3$ per Gm. It is available in capsules; each contains 250 mg. or 100 U. Statistical analysis of the Reference Standard capsule weights revealed that the accuracy of fill is such that 99% of the capsules are within the limits of 0.7% of the stated weight of 250 mg. The average weight is 0.2506 Gm.

Studies carried out by 18 laboratories in the United States and Canada in connection with the development of the new U. S. P. and International Standards demonstrated that 100 U. S. P. Units, as represented by the Cod Liver Oil Reference Standard, equal only 93.4 I. U. Therefore, products assayed against the new U. S. P. Vitamin D Reference Standard may be expected to contain, on an average, 93.4% of the unitage shown when assayed against the last U. S. P. Cod Liver Oil Reference Standard.

Following the practice previously established with Cod Liver Oil Standard, the new Vitamin D Reference Standard is also available for the assay of poultry feed supplements, using the chick assay procedure of the A. O. A. C. Because a greater

quantity of oil is required by this assay, the material is packaged in bottles containing 30 cc. This special package is made available as an added U. S. P. service and is distributed only with the understanding that it is to be used for the chick assay procedure.

A considerable difference exists between the A. O. A. C. Chick Unit and the U. S. P. Unit for vitamin D. Thus, products assayed against the new U. S. P. Vitamin D Reference Standard may be expected to contain, on an average, 75% of the unitage shown when assayed against the last U. S. P. Cod Liver Oil Reference Standard. This difference in value of Units is of great importance both commercially and otherwise. The change is justified on the basis that it represents the establishment of a firm and permanent basis for standardizing the vitamin D content of feeds for domestic animals, especially poultry. Attention is called to the 1950 revision of "Recommended Dietary Allowances for Poultry," published by the National Research Council, which lists the adjustments necessary because of the change of vitamin D Units. In order to differentiate between the present A. O. A. C. Chick Unit and the new Unit which is 33% larger, the term "International Chick Unit" has been adopted for use in the A. O. A. C. method.

The use of the crystalline vitamin as a Standard will now make it possible to express vitamin D content in terms of weight rather than in units, if desirable.

## PURIFIED CRYSTALLINE REFERENCE STANDARDS

This discussion has presented sufficient evidence of the problems involved in preparing U. S. P. Reference Standards of the heterogeneous group. Fortunately, little trouble is encountered in preparing Standards of the crystalline type. On the other hand, recent experience has shown that great difficulties may be encountered even with highly purified crystalline substances, clearly exemplified in the case of Cyanocobalamin Reference Standard.

**Cyanocobalamin.**—Cyanocobalamin (Vitamin $B_{12}$) Reference Standard became necessary for use in the microbiological Vitamin $B_{12}$ Activity Assay of the Third Supplement to the U. S. P. XIV. It was not possible to proceed to any extent with the new Reference Standard until the final details of the assay had been accepted. On the other hand, no difficulties were contemplated because the proposed Standard was to be made from a highly purified crystalline vitamin $B_{12}$. A new problem arose, however, since vitamin $B_{12}$ is required in such minute quantities (0.1 millimicrogram or less per cc. in the microbial assay and scarcely more than 10 μg. per cc. in the spectrophotometric assay). The vitamin is also costly; the current quotation is $325 per Gm. Thus, to provide enough of the crystalline vitamin to weigh accurately even on a sensitive analytical balance was not possible. It was proposed, therefore, that the crystals should be diluted suitably and prepared in the form of tablets, each sufficient for one assay.

Commercial Vitamin $B_{12}$, recrystallized three times, showed 99.7% purity by the solubility method of assay and 99.5% by ultraviolet spectrophotometry. This was diluted with mannitol so that each 100 mg. would contain 50 μg. of the vitamin on the anhydrous basis. The powder was prepared in 20-mg. tablets without the use of lubricant or binding agent.

Spectrophotometric assay of the original mannitol-vitamin mixture agreed with the expected potency, but when the tablets were examined by the same procedure they were found to have an absorbancy 8–10% higher than the original powder from which they were made. Examination showed the presence of what appeared to be filter paper fibers which accounted for some of the additional absorption.

A new lot of vitamin was then prepared, diluted with reagent sodium chloride, and subjected to tests. Reports were soon received which indicated difficulty in obtaining a clear solution of the standard. The slight cloudiness interfered with reading the spectrophotometer. Close examination of solutions of the proposed standard showed glasslike dust particles which apparently arose from attrition action of the harsh sodium chloride on the mortar in which the dilution was made. Filtering the solution to remove the particles did not entirely eliminate the high absorption at lower wave lengths, and this dilution was discarded.

This new difficulty with a Reference Standard which had been expected to provide no problems was most disconcerting, and with considerable resignation a third lot of Standard was prepared. Mannitol was again used as the diluent since, being less harsh than the sodium chloride, it would eliminate the difficulty experienced with the previous dilution. When preliminary tests raised some doubt about its homogeneity, the powder was again mixed and re-examined. Subsequent reports finally indicated that the Standard had fully passed the necessary tests, and official approval was given by the Reference Standards Committee. The Standard was ready for distribution in February, 1952, although it should have been available several months before January 1, when the Third Supplement to U. S. P. XIV became official.

The potency finally accepted for this Standard was 0.53 μg. or 530 millimicrograms per mg.

**Epinephrine and Epinephrine Bitartrate.**—While U. S. P. IX included the first biological assay requiring epinephrine as a Standard, the assay was optional and it was not until 1936 that an official U. S. P. Epinephrine Reference Standard was prepared. Several lots of the Standard have since been issued, and each was checked for conformity to U. S. P. specifications, the most important of which was specific rotation. Each lot was also checked biologically against the previous Standard. There is no International Standard.

Epinephrine base is unstable by nature and difficulty is encountered periodically with the development of a slight color although a loss in biological potency cannot be detected.

In 1947, consideration was given to replacing epinephrine base with epinephrine bitartrate as the U. S. P. Reference Standard. This salt is more stable and because of its higher molecular weight, greater accuracy in preparing solutions from small quantities can be achieved.

While this problem was under study, a report appeared in the literature revealing that the natural epinephrine base generally available in the past, including the base used as a U. S. P. Reference

Standard, contained *l*-arterenol in amounts up to 17%. Since the presence of *l*-arterenol was scarcely detectable in measurements of specific rotation, or by the official biological assay, its presence had never been discerned in the U. S. P. Reference Standard or in official epinephrine of natural origin. It was recognized that the presence of such a large quantity of contaminant in an official U. S. P. Reference Standard could be eliminated by using synthetic epinephrine bitartrate.

Thus the Reference Standards Committee approved the use of epinephrine bitartrate. A lot of this new material was prepared in 1950 and its use was recognized in U. S. P. XIV.

The physical characteristics of epinephrine bitartrate are strong evidence of its purity. Most striking among these are the U. S. P. melting range, specific rotation, and rotation of the separated base determined in an identification test, and a special solubility analysis in ethyl alcohol, an exacting type of assay frequently employed for determining the purity of U. S. P. Reference Standards.

The original solubility analysis experiments in this case led to exhaustive tests of the purity of the proposed Reference Standard. The initial solubility analysis was made with methyl alcohol and, quite surprisingly, appeared to indicate the presence of some impurity to the extent of at least 7%. This did not seem plausible in view of the other data. A portion of the crystals was recrystallized and the remaining mother liquor was then evaporated to dryness; both of these products, as well as a portion of the original material, were subjected to infrared spectrophotometry. The absorption spectra were superimposable over the entire range of 2 to 14.5 mμ, providing striking evidence of the purity of the crystals.

Further study revealed that the discrepancy was attributable to a reaction between methyl alcohol and epinephrine in the course of the solubility analysis, thus invalidating the results. When the analysis was repeated, using ethyl alcohol as the solvent, a purity of over 99% was indicated.

**Histamine Dihydrochloride.**—Histamine Dihydrochloride Reference Standard is actually required by the Pharmacopeia not as a biological reference standard but in the absorbancy assay for Histamine Phosphate Injection. However, this Standard can be used for the Depressor Substances Test for U. S. P. heparin sodium and the antibiotics.

The Standard is a white crystalline powder characterized on the basis of its nitrogen and chloride content, residue on ignition, and melting range.

**Insulin.**—The U. S. P. Insulin Reference Standard is available primarily for manufacturers of insulin and for enforcement agencies. The Standard first became necessary in December, 1941, when the insulin patents expired. The Pharmacopeia immediately established a U. S. P. monograph to provide the standards necessary to insure maintenance of high quality and uniformity of potency. To meet the immediate situation, the Insulin Committee of the University of Toronto offered to give to the Pharmacopeia, for distribution as a Reference Standard, some of the stock of zinc-insulin crystals which manufacturers in Canada and the United ntates had been using. Its potency had been determined in a careful comparison with the International Standard.

Since the total available supply of this lot of crystals was limited, immediate steps were taken to prepare a new lot. The initial plan was to obtain and mix material from several manufacturers and submit it for collaborative study. Further consideration of this proposal indicated several obstacles and once more the Insulin Committee offered its services in the selection and preparation of a substantial quantity of crystals which would again serve as both a U. S. P. and a Canadian Reference Standard. About 500 Gm. of crystals were thus prepared.

A question then came up regarding the moisture content of the crystals and the ability to package the Standard to maintain its uniformity. It was finally decided to have the crystals dried under carefully controlled conditions by Merck & Co., Rahway, N. J. However, the problem of U. S. import duty on this valuable lot of insulin, now arose. After considerable difficulty, the Commissioner of Customs agreed to allow one of the representatives of the Insulin Committee of the University of Toronto to bring the Zinc-Insulin crystals across the border under bond. The crystals were uniformly dried and then returned to Canada.

The newly prepared crystals were subjected to a collaborative study by six laboratories. One collaborator used a special technique, based on the incidence of convulsions in mice, which employed 31 pairs of tests with an average of 425 mice per pair. Another collaborator adopted some modifications of a rabbit assay in which four tests each were made on 20 rabbits. The other four collaborators conformed to the U. S. P. XII method. Statistical analysis of the six reports showed remarkable uniformity, and indicated that the new preparation possessed a weighted geometric-mean potency of 23.03 ± 0.16 I. U. per mg. After consideration of the report, the U. S. P. Insulin Advisory Committee assigned the value of 23 U. to the new material.

**Lanatoside C.**—Lanatoside C Reference Standard is required in the assays for Lanatoside C Injection and Tablets, employing pigeons as the test animal. Because it is a crystalline substance, its preparation offered no particular difficulties and its character was quite satisfactorily defined by its specific rotation.

**Nicotinic Acid.**—Nicotinic Acid Reference Standard is required in the U. S. P. microbiological assay for preparations of Nicotinic Acid and Nicotinamide, first officially recognized in the U. S. P. XII Bound Supplement.

No unusual difficulties have been encountered in preparing several lots of this Standard. The acid is recrystallized and the combination of its melting range and assay serve to guarantee its purity. In addition, it is checked for loss on drying, residue on ignition, and the presence of chloride and sulfate.

**Ouabain.**—As stated above, ouabain was used in the U. S. P. IX, 1916, as the Standard in the optional biological assays for digitalis, strophanthus, and squill. Reagent grade ouabain was used for this purpose and complete specifications were given for it in the reagent section of the Pharmacopeia. Since pure ouabain was distributed for reference standard purposes by the Bureau of Chemistry of the U. S. Department of Agriculture from about 1926, the U. S. P. did not recognize an official Ouabain Reference Standard until 1936. The first Standard con-

sisted of ouabain which had been crystallized from alcohol and contained only about 11% water. Commercial ouabain is crystallized from water and contains about 20% water. When new stocks were required in 1946, a question arose as to whether the Reference Standard should have a higher water content. It was finally decided that the original Reference Standard should be duplicated to avoid confusion.

**Riboflavin.**—Riboflavin Reference Standard is required for the official microbiological assay.

Four different lots of this Standard have been prepared without difficulty since it was first made available in 1941. Melting temperature and specific rotation definitely characterize it, but each lot is also compared against the previous Standard by the U. S. P. microbiological assay. At present the Standard is also used in the official fluorometric assay required for the dosage forms of this vitamin.

**Testosterone Propionate.**—Testosterone Propionate Reference Standard was prepared in 1945 for use in a collaborative study of assay procedures with the hope of recognizing testosterone propionate preparations in U. S. P. XIII. However, it was five years before this hope materialized in the U. S. P. XIV biologic assay for testosterone propionate injection. The Standard represents a composite of three lots of testosterone propionate, each of which was carefully identified before blending. The final mixture was subjected to examination by several laboratories for melting point, specific rotation, and similar exacting tests.

**Thiamine Hydrochloride.**—Thiamine hydrochloride was given its first official U. S. P. recognition by the Second U. S. P. XI Supplement in 1939. A chemical assay which did not require a Reference Standard was specified. However, the Supplement included a biological assay for preparations of thiamine hydrochloride for which a Reference Standard was needed.

The need for this new Reference Standard and an assay for vitamin $B_1$ preparations was pointed up by action taken by the American Medical Association Council on Pharmacy and Chemistry which ruled that all products containing vitamin $B_1$ potency and recognized by the Council must be assayed in terms of the International Unit. Since the stock of the International Standard was insufficient, the Pharmacopeia undertook to provide the required Standard.

A clay adsorbate of vitamin $B_1$ similar to the International Standard was selected and had actually been purchased in 1936 when the U. S. P. received an announcement in January, 1937, that the synthetic vitamin $B_1$ would soon be generally available. The adsorbate was discarded at once in favor of the crystalline vitamin which became the first Thiamine Hydrochloride (vitamin $B_1$) Reference Standard. However, two years elapsed before it was released in 1939. This first lot represented material obtained from two sources and recrystallized. Several replacement lots have since been prepared.

**Tubocurarine Chloride.**—The U. S. P. XIV head-drop assay for Tubocurarine Chloride Injection is a cross-over procedure requiring a Reference Standard. Two lots of tubocurarine chloride were finally selected from several examined to provide the Standard. While both lots represented highly purified material it was found necessary to recrystallize the composite twice before the product was satisfactory.

The purity of the Reference Standard is definitely established by its melting range, specific rotation, chlorine content, residue on ignition, and by a test for chloroform soluble substances.

**U. S. P. Reference Standards Available.**—A complete list of Reference Standards which are available is given in Table I.

TABLE I.—U. S. P. REFERENCE STANDARDS AVAILABLE

| Standard | Size |
|---|---|
| Ascorbic Acid | 1 Gm. |
| Calcium Pantothenate | 200 mg. |
| Cholic Acid | 2 Gm. |
| Choline Chloride | 2 Gm. |
| Corticotropin (ACTH) (approx. 0.1 U/Tab.) | 25 tab. |
| Cyanocobalamin (vitamin $B_{12}$) (0.53 μg./mg.) | 400 mg. |
| Diethylstilbestrol | 200 mg. |
| Digitalis | 3 Gm. |
| Digitoxin | 50 mg. |
| Digoxin | 50 mg. |
| Epinephrine Bitartrate | 200 mg. |
| Ergonovine Maleate | 20 mg. |
| Estradiol | 20 mg. |
| Estradiol Benzoate | 20 mg. |
| Estrone | 75 mg. |
| Heparin (approx. 48 U/Tab.) | 25 tab. |
| Histamine Dihydrochloride | 250 mg. |
| Homatropine Methylbromide | 100 mg. |
| *l*-Isoleucine | 250 mg. |
| Lanatoside C | 50 mg. |
| *l*-Leucine | 250 mg. |
| *l*-Lysine Monohydrochloride | 250 mg. |
| Melting Points—6 standards; each | 5 Gm. |
| Menadione | 500 mg. |
| *l*-Methionine | 250 mg. |
| Methyltestosterone | 50 mg. |
| Nicotinamide | 500 mg. |
| Nicotinic Acid | 500 mg. |
| Ouabain | 100 mg. |
| *Para*-aminobenzoic Acid | 500 mg. |
| Penicillin G Sodium | 150 mg. |
| *l*-Phenylalanine | 250 mg. |
| Posterior Pituitary (approx. 9.4 U/Tab.) | 20 tab. |
| Potato Starch | 100 Gm. |
| Progesterone | 20 mg. |
| Pyridoxine Hydrochloride | 200 mg. |
| Riboflavin | 500 mg. |
| Sulfanilamide | 25 Gm. |
| Testosterone Propionate | 20 mg. |
| Thiamine Hydrochloride | 200 mg. |
| *l*-Threonine | 250 mg. |
| Thyrotropin Substance (approx. 1 U/Tab.) | 20 tab. |
| *l*-Tryptophano | 250 mg. |
| Tubocurarine Chloride | 250 mg. |
| Tyrothricin | 200 mg. |
| *l*-Valine | 250 mg. |
| Vitamin A (1 capsule = 2500 U) | 24 cap. |
| Vitamin D Capsules (1 capsule = 100 U) (U. S. P. rat assay) | 48 cap. |
| Vitamin D Oil (A. O. A. C. chick assay, 1 Gm. = 400 U) | 30 cc. |

# International Biological Standards and the World Health Organization*

By A. A. MILES

IN DISCUSSIONS about international work on biological standards, the phrase "philosophy of the W. H. O. Committee" is sometimes heard. At first sight the word "philosophy" seems much too grand for the very empirical way the Expert Committee on Biological Standardization has carried out its international work; but a second thought is more disturbing. One of the criticisms leveled at a certain kind of philosopher is that he starts with a set of *a priori* propositions, which appear to have been dug up from his inner consciousness and from which he proceeds very arbitrarily to set the world to rights. This second thought is not necessarily in the minds of those who use the phrase, but it raises the speculation that to users of biological standards the proceedings of the Expert Committee might sometimes appear to be as arbitrary as those of a philosopher who makes the facts of Nature fit his preconceptions, instead of building his conceptions from the facts. There are good reasons behind most of the Expert Committee's decisions, but it must be admitted that these reasons are often not published in detail, and those that are published, in the Bulletin of the World Health Organization and in the W. H. O. Technical Report Series, do not receive as wide publicity as they should. If there is a philosophy behind the handling of international standards, it does not float, well insulated from reality, in the realms of pure thought, but like all good philosophies, is rooted in everyday experience and applied with a liberal dash of compromise.

## BACKGROUND

The relevant background of the international biological standards is compounded of a little history and a description of their organization, but consists mainly of the principles behind the establishment and use of the standards.

The earliest biological standards were immunological. Ehrlich's diphtheria antitoxin, made in Germany in the 1890's, and Rosenau and Anderson's tetanus antitoxin, made in the United States in 1908, both eventually became international standards. The diphtheria antitoxin standard was created in 1922 by an International Conference called by the League of Nations Health Organization. Other conferences in 1923 and 1925 established standards for digitalis, insulin, pituitary (posterior lobe) extract, neoarsphenamine and sulpharsphenamine. After this, the work was entrusted to a Permanent Commission on Biological Standardization, and two world centers were created for the distribution of the standards—namely, the State Serum Institute in Copenhagen, which was given the custody of the immunological standards, and the National Institute for Medical Research in London, which looked after the remaining pharmacological standards. By 1939, 35 international standards had been established. During the war when Denmark was overrun by Germany, London looked after the serum standards. International work on standards did not cease; three provisional standards—vitamin E, heparin, and penicillin—were created in the war years. At the end of the war, the work of the League of Nations Health Organization and the international biological standards were taken over by the World Health Organization. The immunological standards were returned to Copenhagen, and the 3 war-time provisional standards were given full international status (10–12).

The Permanent Commission on Biological Standaridization, however, was not reconstituted in the form it had under the League of Nations Health Organization; instead, an Expert Committee on Biological Standardization was set up with the same status as other W. H. O. advisory committees. As the functions of the W. H. O. have gradually crystallized, the Expert Committee system, as far as it concerns standards, has now the structure and world relationships outlined in Fig. 1. In the different member states, persons expert on various aspects of standardization or on the substances standardized are appointed to an advisory panel.

The Expert Committee on Biological Standardization is newly formed each year and consists of seven or eight members drawn from the advisory panel. Continuity is formally assured by the fact that Dr. W. Aeg. Timmerman, who is in charge of the Division of Therapeutic Substances of the W. H. O., is *ex officio* secretary of the Committee. Problems and suggestions are brought by the committee members and many are referred to the committee by other expert committees of the W. H. O. Thus, the Joint WHO/FAO Expert Group on Zoonoses and of the Office International des Epizooties recently asked the standardization committee to adopt veterinary biological standards. A start has been made with Q fever and *Brucella abortus* diagnostic antisera, and a standard for avian P. P. D. The committee is in constant liaison with the Expert Committee on the Unification of Pharmacopoeias. For the most part, standards already exist for the majority of the biological substances described in the Pharmacopoeia Internationalis (Ph. I.). The decision to include a monograph on a sub-

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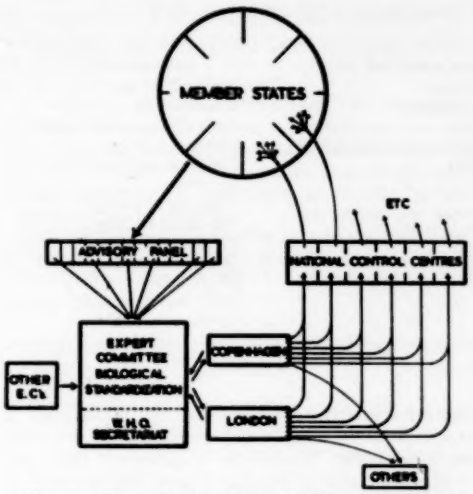


Fig. 1.—The relationship of bodies dealing with biological standards under the World Health Organization.

stance for which there is as yet no international standard (e. g., globin zinc insulin or protamine zinc insulin) means that the standardization committee must provide standards for methods of assay recommended in the Ph. I. appendices.

The recommendations of the Committee are ratified by the Executive Committee of the W. H. O. and the practical work is carried out chiefly by the two international centers, the State Serum Institute in Copenhagen and the National Institute for Medical Research in London. These centers have two main functions—the establishment of standards and their distribution when established.

For the global distribution of standards, the two centers rely on the system of National Control Centers, which were set up by the League of Nations Health Organization in each member state, not only to relieve the international centers of the administrative difficulties of getting standards to *bona fide* users in the various countries, but also in the hope that each center would provide national standards measured in terms of the International Standards, and further the cause of proper biological standardization in those countries. These hopes have been fulfilled in a number of countries.

The organization of international standards is well adapted to investigate and integrate world opinion, and to ensure full use of the standards. However, the Committee's work usually lags behind recent advances in the field of biologically active substances; and in the very real need for speedy action, it may not be possible to give full consideration to all relevant views about a given standard. Speed is necessary because when a new substance comes into general use, both clinicians and the pharmaceutical industries want a measure of potency, and if there is no national or international lead in this matter they set up their own specifications or standards. This may be a good thing, but it often leads to more difficulties than it solves, because conflicting and perhaps invalid measures of potency may come into general use and the resulting confusion takes years to overcome.

Both biologists and biometricians have been very successful in the last twenty years in proselytising the ideas of valid bioassay in terms of material standard preparations, and their success is reflected in the work of the standards committee, which has done a great deal of proselytising on its own account. Figure 2 records the increasing number of international standards established. There was naturally a lag during World War II, but after that the graph becomes steeper than ever before. There are fewer replacements of standards that are exhausted. Mere numbers however are misleading. Whereas with an entirely new standard, potency is largely a matter of agreement on an arbitrary figure, a replacement standard preparation must be assayed with the greatest possible care, by laboratories all over the world, against the existing standard preparation to ensure that the new unit of potency is truly equivalent to the old. The most impressive curve is that for the new standards, whose establishment is already authorized by the committee, and which are underway. There are nearly 40 of these new standards (see Table I); those for the new antibiotics are relatively simple, because there are only one or two sources for the material in each case; but with hormones and the various antisera, for example, of which there are already so many informed users of the substance all over the world, agreement about a new standard entails a great deal of labor and collaborative assay. This increase in the speed of establishing standards may explain why in the old League of Nations Bulletin a whole volume is devoted to the report on a new standard, with papers from each participant published *in extenso;* whereas nowadays a short paper summarizing the collaborative assay is all that appears.

## THE EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

When the Expert Committee on Biological Standardization took over from the League of Nations, it was at first guided by Dr. R. Gautier, who had been secretary of the Permanent Commission, and it inherited a number of general guiding principles, which it has subsequently put into practice. Per-

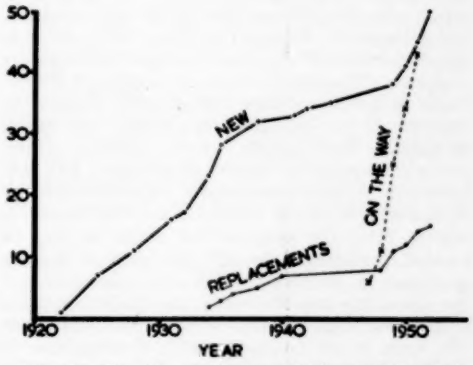


Fig. 2.—Graphs of the cumulative totals of international biological standards; established, replaced by new preparations, and in preparation.

TABLE I.—INTERNATIONAL BIOLOGICAL STANDARDS IN PREPARATION (1952)

| Antibiotics | Pituitary Hormones | Pharmacological, Etc. | Immunological |
|---|---|---|---|
| Aureomycin | Growth | Dimercaprol | Avian P.P. D. |
| Bacitracin | Thyrotrophin | Hyaluronidase | Cardiolipin |
| Chloramphenicol | | Male fern | Brucella abortus serum |
| Dihydrostreptomycin | | Melaminyl trypanocides (3) | Cholera vaccine |
| Terramycin | | Thrombin | Cholera antigens (2) |
| | | | Lecithin |
| | | | Opacity standard |
| | | | Pertussis vaccine |
| | | | Proteus sera (3) |
| | | | Q-fever serum |
| | | | Rh sera (3) |
| | | | Salmonella sera (8) |
| | | | Tetanus toxoid |

haps the most important principle is that standards must be tailored, as it were, for world use. For example, although advanced countries may have perfected methods of chemical or physical assay of a substance, less technologically advanced countries may prefer a simpler biological assay, so that a standard must be provided for them, although it may have become obsolete for others. Again, the standard preparations must be kept as simple as possible to ensure that their use does not demand unnecessarily special skill or apparatus. For example, if a substance is highly active, like vitamin $B_{12}$, it must be diluted with an inert substance, so that in powder or tablet form its potency can be accurately determined by weighing on an ordinary precision balance. Another most important principle, which is considered in detail below, is that the World Health Organization does not insist on any one method of assay except in special circumstances; thus the standard preparation must as far as possible be suitable for all the reputable methods of assay in use throughout the world. Moreover, standards must be established so well that no foreseeable ground is left for complaints that some precaution or other was not taken; a custodian of standards must be able to feel, whenever some anomalous assay is debated, that he is justified in insisting that all other probable sources of error are investigated before the standard is doubted.

In the establishment of a standard, international agreement on many points is required. The standards committee decides to set up a standard as the result of representations that, either for research or for the control of drugs containing the substance, agreed methods of assay and an agreed potency are desirable. The international center either in Copenhagen or London is then authorized to explore the possibility of a standard preparation. The center in London, for example, usually convenes an advisory conference of experts in Britain. The conference draws up a questionnaire about the form the standard preparation should take, its degree of purity, etc.; the purposes for which it may be wanted, particularly the different methods of assay that may be practiced in different countries; and the size of the unit of activity, especially in relation to any existing standard preparations and units. The form of the proposed standard preparation is decided from the answers to this questionnaire. With most substances, the preparation is a blend of samples from different laboratories and different countries, partly to ensure that the preparation is representative of all such preparations wherever made, and often because the substance at this stage is expensive and no one contributor can be asked to undertake the labor of making the whole standard preparation. In some cases, a standard preparation is already well established, either officially or unofficially, and can be taken over. Thus the Armour and Co. preparation La-I-A was established as the first International Standard for Corticotrophin, and the existing National Institutes of Health Standard for Scarlet Fever Antitoxin has been adopted as the basis for the new International Standard.

The next stage is to devise a collaborative examination of the material. Sometimes preliminary examinations of the samples from the bulk batch are made, but the essential feature is an examination of the material *as dispensed* (usually dried, in ampuls, under an atmosphere of nitrogen) in the form of the final standard. Only in this way is it possible to avoid justifiable criticisms that may later be made that a standard preparation, tested in bulk, changed essentially during the manipulations necessary for distributing it in the final form.

After the collaborative examination of the standard, the Expert Committee has to decide upon the size of the unit of potency. When there are different units in existence, this may require a good deal of negotiation and some final compromise. Finally, a memorandum on the history, form, and use of the standard is written for distribution with the preparation. It is important here to note that the international standard preparations are intended only to be vehicles of potency, either therapeutic or toxic. In this respect they differ from the U. S. P. Reference Standards, which are not only vehicles of potency but in as many respects as possible resemble the substance as described in the U. S. P. monograph. In other words, they are exemplary standards, and if current preparations have a certain high potency the standard has approximately the same potency. The international standards are not exemplary in this respect. In the international view, the actual potency of the standard does not matter provided it is active enough to be used in assay and that the inert material does not interfere with the specific activity of the preparation in the test animal. Thus, although the potency of a standard might only be 5 U./mg., purely as a vehicle of potency, it is considered suitable for assay of test preparations that may have 50 or 100 U./mg. Another point is that

the potency assigned to the standard is, wherever possible, not limited to a particular method of assay. Methods of assay are only recommended in the memorandum.

In the replacement of a standard, the procedure is similar except that the collaborative assay is far more laborious, since, the unit of potency in the new preparation must be the same as that in the old. In both collaborative examinations, results by the various methods of asssay in general use are welcomed, both as valid contributions to the final estimate of the potency of the material, and as evidence of the performance of the standard under various conditions.

## THE EXPLOITATION OF BIOLOGICAL STANDARDS

Turning now to the more general purpose upon which standards are established and accepted, we may note that they flow from the very nature of biological assay and are best discussed by considering biological assay in general. Some of the discussion that follows may appear elementary, and a great deal of it has already appeared in other publications (1–12). But it is offered with the excuse that many of the concepts in biological standardization are perhaps so simple that their implications are missed by many who use biological standards; and it is better to be elementary than obscure.

**Standard.**—It is important to agree upon the meaning of the terms 'biological standard' and 'biological substance'. A 'standard' should not be confused with a 'specification'. A pharmacopoeial monograph may in common parlance "set a standard" but it is in itself only a specification of requirements. In this context the word 'standard' should be reserved for a fixed material thing, whether it is a physical standard like the standard meter in Washington or the ampul of digitalis powder held in New York as the U. S. P. Reference Standard for digitalis. By "biological substance" is meant, in this context, something with biological activity that cannot be specified fully by chemistry and physics.

**Potency.**—Biological potency is by definition a measure of activity in some biological system. The biological system may be a whole animal, like a cat or pigeon; part of an animal, like a suspension of red cells or an isolated guinea-pig gut; or a culture of bacteria. But whatever it is, our first idea of potency is in terms of action on a biological system. This is natural but unfortunate, because most of the trouble in assay arises from the concept of potency as the amount of substance that has a certain effect on a certain biological system. For example, the potency of digitalis extract used to be defined as the dose killing a cat during intravenous injection, and was measured in 'cat units'. But it would not be unfair to translate 'cat unit', as measured in a given laboratory as follows: "A cat unit is the mean of a certain range of lethal doses of a particular preparation of digitalis that happened to be killing the twenty miscellaneous cats that were obtained from the city pound last weekend"; and this is not impressive as a reliable measure of potency. When we come to define a unit of activity of some ill-defined biological substance, we quite rightly attempt by analogy with chemistry and physics to describe the unknown in terms of the known. But as between digitalis and the cat, the 'cat-unit' defines the digitalis, not in terms of the known, but only in terms of the familiar, because, except in the broadest way, we are quite ignorant of the causes that make one cat differ from the next in its response to the mixed digitalis glucosides.

It cannot be denied that with certain drugs, the workers in a given laboratory, who have perhaps carefully selected and bred a uniform stock of animals or microbes (i. e., a uniform biological system) can get reproducible results in terms of animals or microbial response. However, this measure of potency by direct assay is largely private, and can often be communicated to other workers only by great labor, including training workers in the laboratory, giving them the stock strain of animals, the animal diet, and so forth. But even with precautions like these, it is impossible to be certain that the biological system finally used in two or more laboratories is exactly the same in each. There is, in fact, only one simple way of testing how far they are the same, and that is by measuring their response to a stable standard preparation of the drug, even though we may not, as chemists and physicists, know exactly what the drug is. That is to say, we have a largely undefinable biological system and a relatively unknown substance we wish to assay; and in seeking for some point of reference in this system, we choose not the thing about which we have or think we have the most knowledge, but the thing we can keep stable most easily; this proves to be a preparation of the substance we are studying.

Once we have a stable standard, the measurement of potency is simple in theory, because we assign unit potency to a given weight of the standard preparation, and measure all unknowns in terms of the unit.

## DIFFERENT KINDS OF BIOLOGICAL ASSAY

Three kinds of biological assay can be distinguished: direct, "orthodox," and ideal (Table II); and of these we are here chiefly concerned with the second.

**Direct Assay.**—Potency is measured directly in terms of a biological effect. Assays of this kind are rather restricted in scope, because the biological system is, in a last analysis, indefinable. We have to rely on this method when it is impossible to devise a stable standard. It happens particularly with

TABLE II.—THE THREE KINDS OF BIOLOGICAL ASSAY

| Reference Point | "Standard" | Measure of Potency |
|---|---|---|
| Biological system | Biological specification (necessarily incomplete) | A dose-range (restricted) |
| Active material, chemically undefined | Stable preparation | Unit ≡ weight of unique preparation (universal) |
| Chemically defined substance | Chemical specification | Weight (universal) |

substances used in active immunization—vaccines, toxoids, and other antigens. Some progress has been made toward establishing standard diphtheria and tetanus toxoids, and standards for cholera and pertussis vaccines. It is relatively easy to make a stable preparation of either of the toxoids; the chief difficulty at present is to decide the form the standards shall take, because they have to serve for the assay of several forms of tetanus and diphtheria prophylactics. With bacterial vaccines also, there is usually no difficulty in making a stable preparation; but it is often doubtful whether immunity induced in the test animal by the vaccine, and the bacterial infection used to test the immunity, are sufficiently representative of the natural infection in man and the kind of immunity needed for protection against it.

For these reasons, bacterial vaccines are often specified, most inadequately, in terms of numbers of contained bacteria, and the strain of bacterium to be used in the preparation is specified as having the properties generally believed to be associated with immunizing potency. Toxoids are directly assayed in terms of animal response, but the response at least can be measured in terms of standard antitoxins. Vaccines like those against smallpox and yellow fever, which contain viruses in an infective state, cannot be assayed against standards, owing to our inability to preserve viruses without deterioration of infective titer, or to relate with any certainty viral antibody response (which would be measurable in terms of a standard antiserum) to their immunizing potency. Like toxoids, virus vaccines are specified by "minimum requirements" that include the estimation of a range of effective doses in the test animal. Now control by minimum requirements depending on direct assay in an (ultimately) indefinable animal is justified only when the substance can be administered to man in many multiples of an effective clinical dose. It is sufficient, for example, to demand that diphtheria prophylactic should satisfy minimum requirements for immunizing potency in the guinea pig, because these requirements can be devised to take into account the wide variability of laboratory animals. If in any one test the immunizing potency is by chance determined on a group of relatively unsusceptible animals, the consequent under-estimation of potency will result only in an innocuous overdosage of man with the prophylactic, and if the potency is overestimated on relatively susceptible animals, the dose in man will still be adequate.

Direct biological "assay" though successful in practice, should always be regarded as a second-best method and should be replaced whenever practicable by assay against standards. It is not justifiable to use it for dangerous drugs, because the wide range of potencies indicated by the animal response cannot be allowed for by overdosage with possibly weak preparations. Finally, it must be emphasized that the "unit" system of potency notation should never be used for drugs so assayed (10–12).

**Orthodox Bioassay.**—Here the assays are made against biological standards, where the potency ratio is measured in terms of a unique preparation of a master standard by comparing standard and test preparation simultaneously, if possible.

**Ideal Bioassay.**—Here the activity of the preparation is compared with that of a defined chemical substance and for obvious reasons the bioassay is possible only with substances of low molecular weight that are within the present scope of chemical analysis. There need be no agreed standard preparation of the chemical. A specification which, as in some pharmacopoeial monographs, defines the biologically active substance in chemical terms, provides for a valid standard wherever chemistry and physics are competently practised. It may be convenient to maintain an authentic preparation of the substance for use in assay (we have, for example, reached this stage with chloramphenicol, an established "fine chemical," whose potency is sufficiently defined by the weight of the drug) but the authentic preparation is a *chemical*, not a biological standard.

## THE REQUIREMENTS FOR VALID BIOLOGICAL ASSAY

Assay in terms of a stable biological standard yields a ratio of potencies of the standard and the unknown test preparation. If the ratio of potencies of the standard and of the test preparation is to be regarded as a valid expression of the potency of the test preparation, there are certain assumptions that must be made about a standard preparation. They are as follows: (*a*) that the standard preparation is stable; (*b*) that the biological test system represents a random sample of a population of test-objects; (*c*) that the response of the biological system to the standard and test preparations is due to the active principle it is desired to measure; (*d*) that the ratio of potencies so obtained is independent of the biological system used; (*e*) that the standard preparation contains neither impurities having a specific activity resembling that of the active principle, nor substances which in any way modify the behavior of the active principle; (*f*) that the biologically active constituent in the standard preparation is homogeneous; (*g*) that the active substance assayed is the same as the active substance in the standard.

**Stability of the Standard Preparation.**—The first assumption by itself needs no further elucidation. It is however, worth while to note the methods of estimating stability. Most of the international standards are held at −10° in the dry state, in an atmosphere of $N_2$, and it is our experience that they maintain their potency unimpaired. On this basis we can predict with some confidence the success of preservation by these methods, particularly with modern methods of drying from the frozen state at carefully controlled temperatures, which are efficacious than the cruder methods used for the earlier standards. But the analogy is not enough; it immediately raises the question: "How do we know that these earlier preparations have lost no potency?" For if a preparation has been preserved by the best method available, any test of maintained potency during storage must be in terms of the preparation itself, which is absurd, or of a similar preparation which may be equally subject to deterioration. Now the proper way out of this impasse is usually taken, but often by mere accident of numbers, and seldom as a clearly recognized necessity. We must at the very least copy the pre-Marconi navigator who, in the absence of a direct astronomical observation, takes his time from chronometers and checks any deteriora-

tion in their behavior by having three of the best that money can buy. Let us be even more certain, and take six preparations; then if a standard preparation always matches the potency of five other similar but different preparations preserved in the dark and cold at different institutes, it is, statistically speaking, far more likely that all six have maintained potency than that all six have declined, strictly in parallel, over a number of years. With many International Standards, their repeated comparison with national biological standards constitutes a statistical check of this kind.

It is well to remember, however, though in rejecting direct assay as imperfect we have dethroned the biological response from first place in biological assay, yet it still plays the part of a 'hidden' standard. The proof of the stability of the standard preparation over many years, by testing at intervals against other standard preparations of the same substance, is usually undertaken only when there are clear indications that something is wrong with the standard; and that indication is commonly a report that the animal response to the standard has changed. The response to a drug of an animal stock maintained in a laboratory is not reliable as a measure of potency, as we have seen, because it is impossible to specify the conditions under which the stock will keep its presumed constancy in this respect. But within one laboratory, where the workers are familiar with their animal stock and its breeding and feeding, and are continuously performing a certain type of assay, their combined experience of the standard in relation to their animals, though it is largely incommunicable, constitutes a valuable check on its potency.

**Biological System a Random Sample.**—The relative activity of the 'unknown' material is determined by a parallel test on a similar biological system. The parallelism is ensured as far as possible by comparing the 'standard' and the 'unknown' at the same time on the same animal or animals, as in the assay of tuberculins; or by successive alternation of standard and unknown in the same animal or animals, as in the assay of posterior pituitary hormone or insulin. Most commonly, however, a batch of animals, a preparation of blood cells, or a culture of bacteria is divided into two sets of test objects, and the titration of standard and unknown is performed on them simultaneously. The validity of this comparison depends on how far the biological test material represents a random sample of the population of test objects (animals, bacteria, etc.) employed in the assay.

**Desired Effect Is Measured.**—This means that we are really measuring the active principle in which we are interested and not some other activity. If this is true, it follows that the biological effect measured in the assay need have no relation to the required clinical effect in man. Thus vitamin $B_{12}$ intended for the treatment of anemia in man is validly assayed by its capacity to promote the growth of *Lactobacillus casei*, and tincture of digitalis by its capacity to kill guinea pigs on slow intravenous injection. It also follows that potency need not be measured by any one method. Provided that the assay is comparative, and designed so that a valid measure of activity is obtained, the choice of method is dictated purely by convenience and economy. The validity of the assumption depends on further assumptions about the nature and the state of the active stuff in the standard and test preparations, particularly in relation to its actual or presumed homogeneity.

**Ratio of Potencies Independent of Biological System Used.**—Provided that the biological system is susceptible to the specific activity measured, only consideration of money (and perhaps personal dignity) should prevent the biologist using, for example, elephants, emus, or elks for the assay of ACTH.

**Homogeneity and Similarity of Standard and Test Preparations.**—It is obvious that the standard preparation should owe its activity to one substance only and there should be neither inhibitors nor enhancers of its activity in the impurities (*e*). The word homogeneity as applied to the standard preparation (*f*) can have two meanings. It is of course important that the standard is homogeneous in the sense that there is no undue variation in each portion of the standard preparation as distributed in separate containers for the purposes of issue as a standard. But in this connection a more fundamental homogeneity is intended, in the sense that all the specific activity is due to one compound or one species of macromolecule and not to a family of compounds having similar biological activity.

The last assumption (*g*) is the "hypothesis of similarity" which in fact demands that the qualities of the standard discussed under (*e*) and (*f*) are shared by the test preparation.

These assumptions are stated in a practical form. Many of them can be derived more formally by an examination of the fundamental assumptions of biological assay. For example, Jerne and Wood (9) state them as follows:

(*a*) The differences between responses in the several dose groups of an assay are wholly caused either by differences in dosage or by random sampling.

(*b*) The expected response $U$ is a determinable function of the dose $z$, so that $U = f(z)$ where $U$ is a single-valued function of $z$ over the range of doses used.

For standard preparation $S$,

$$U_s = f_s(z_s)$$

For test preparation $T$,

$$U_t = f_t(z_t)$$

If $z_s$ and $z_t$ are doses giving $U$, the potency ratio is $z_s/z_t$

(*c*) The response to both standard and test preparation is due solely to the same active principle, unmodified by any other substance that may be present in the preparation. If $z_s/z_t$ is to be valid over a whole range of doses, $z_t/z_s$ must be independent of $U$, the conditions of assay, or the biological system used, i.e., $f_s(z_s)$ and $f_t(z_t)$ are the same.

When these conditions are fulfilled, then we have in an assay a series of dose-responses to the standard preparation and a series to the test preparation. In nearly all cases, these responses can be transformed into quantities which, when plotted against log dose, give linear dosage-response slopes. In a valid assay these two slopes are parallel, so that the potency ratio is independent of the response level. If the lines diverge, the result of the assay is said to have a restricted validity. This restriction as the biometrician puts it is merely that potency ratios are valid if measured at the same level of response

each time. But in practice it is a formidable restriction, because in order to achieve it every time we have to specify our biological system, and that, as we have seen, we cannot do with any precision.

The formal biometrician has in fact landed us in the ideal assay, like that of determining biologically the amount of penicillin G in a tablet, or the amount of histamine in a tissue extract. But in the hard practical world we have all kinds of deviations from the ideal and we have no right to assume that either standard or test preparation is pure (Fig. 3). Suppose that the first usable crude standard for, say, an antibiotic contains (although we do not know it) some inert impurity and two distinct molecular species ($\alpha$ and $\beta$) of the antibiotic—as distinct as streptomycin and mannosidostreptomycin (white and cross-shading in the figure). Suppose also that the crude preparations assayed against this first standard also contain only $\alpha$ and $\beta$ and inert impurity. If, as at the top of the figure, the proportions of $\alpha$ to $\beta$ were constant, the hypothesis of similarity holds and the assays are valid. But the likelihood of other proportions and even more complex mixtures makes any constant assumption of the hypothesis unwarrantable. It would be better to assume in all cases that until it is *known*, both standard and test preparation are heterogeneous. With the next advance in purification it may be possible to make a standard consisting of one molecular species, but it may still be necessary to use it in the assay of heterogeneous preparations. How far are we justified in making the assumption that we must expect heterogeneity until we are certain of homogeneity? In other words, active biological substances of large molecular weight (because it is these we usually have to specify by biological assay) are likely to be families of similar molecules and not unique substances. Among substances for which standards already exist, we know for example, of families of D vitamins, of substances with vitamin A activity, of several sex steroids, probably several heparins, and a whole village of families in the antibiotics—the penicillins, the polymyxins, the streptomycins, the bacitracins, and the gramicidins. Among the antitoxins it appears that tetanus antitoxin, and therefore perhaps the tetanus toxin itself, is heterogeneous in this sense, and there are several related tuberculins. There is little doubt that a search would reveal heterogeneity in other immunological standards (though heaven forbid that anyone should disturb the *status quo* by trying to do so).

This very justifiable expectation of heterogeneity, to say nothing of the possibility of synergists in impure preparations (e. g. the presence of thyrotrophin in our proposed growth hormone standard) of course plays havoc with the principle that pure standards must be used in assay, and has led many to advocate that the establishment of biological standards should be deferred until the active principle can be defined with fair accuracy. In my opinion this is a mistaken policy. The standard should be a good deed shining in a naughty world of impure substances; it is better to have a grossly heterogeneous standard than none at all. By all means get the cleanest preparation available at the time, but establish the standard, always supposing there is a demand for it, as soon as there is a fair prospect of making a stable standard preparation.

I would defend this view on the grounds that a standard is a research tool, particularly for the detection of heterogeneity, as much as it is a vehicle of potency for a presumably single substance. And to promote its usefulness in this direction, not only should it be established early, but its use should not be confined to any one method of assay or to any one biological system. These are extremely important points, because there are two main ways in which heterogeneity declares itself quantitatively in assay against a standard; first, change in potency ratio with change in biological system; and second, nonparallelism of dosage-response. The discovery that cod-liver oil was much more potent than irradiated ergosterol in promoting calcification of bone in chickens, as compared with assay in the rat, led to the recognition of a vitamin D in the oil that differed from calciferol. Had the assays been confined to the rat, we might still be ignorant of the distinction between $D_2$ and $D_3$. Again, if the international standard for tetanus antitoxin had always been used with a single toxin, its heterogeneity might still be unrecognized. We have a more modern instance in penicillin, where the change from *Staph. aureus* to *B. subtilis* by Schmidt, Coghill & Ward in 1945 led, through the variable *subtilis/aureus* potency ratios, to the definition of the family of penicillins.

Table III summarizes three assays, carried out by my colleagues Dr. J. H. Humphrey and Mr. J. Lightbown, that illustrate the effect of change in the biological test system when test and standard substances are known to differ. The first is the now classical difference in potency ratio of penicillins G and K on *Staph. aureus* and *B. subtilis*. In the second, the "smooth" *B. subtilis* was our standard laboratory strain, and the "rough" a colony variant isolated from the edge of an inhibition zone; even with this relatively small change, there is a 30% change in potency ratio. The third shows how two strains belonging to the *subtilis* group of bacilli can affect an estimate of potency of dihydrostreptomycin in terms of streptomycin. As an example of change in slope of the dosage-response line, we may take Fig. 3 in Reese and Eisenberg's (13) paper on polymyxin assay, where nonparallelism of the

TABLE III.—CHANGE IN POTENCY RATIO OF WEIGHTS OF HETEROGENEOUS ANTIBIOTICS WITH CHANGE IN THE ASSAY BACTERIUM

| Standard Preparation | Test Preparation | Bacterium Used | Potency Ratio |
|---|---|---|---|
| Penicillin G | Penicillin K | *Staph. aureus* | 2.63 |
| | | *B. subtilis* | 1.00 |
| Penicillin G | Penicillin X | *B. subtilis* ("smooth") | 0.38 |
| | | *B. subtilis* ("rough") | 0.26 |
| Streptomycin | Dihydrostreptomycin | *B. subtilis*, (I. C. I. strain) | 1.00 |
| | | *B. subtilis*, (Marburg strain) | 0.35 |

dosage-response lines in two preparations of polymyxin was their reason (which proved to be well founded) for suspecting that the preparation contained more than one molecular species of polymyxin.

A similar deduction (that there is a fundamental dissimilarity in the two preparations) can be made from some of the Expert Committee's work on standards for diphtheria toxoid. It was hoped that a purified formol toxoid would serve as a standard for all preparations of toxoid, purified, crude, or mixed with adjuvants like aluminium phosphate. However, purified toxoids give a shallow dose-response line, adsorbed toxoid a steep one, and assay of one in terms of the other, it appears, is impracticable because it is invalid.

## THE THREE AGES OF A STANDARD

These examples illustrate the twofold use of standards established during the research and development period of a biological substance, which is a time when biological standards are necessary if there is to be any common ground in measures of potency. There are three ages of a biological standard, of which this rather turbulent first age in the research workers' laboratory and then in pharmaceutical industry is infancy and early manhood. The second is its successful middle age, when it gradually ceases to be necessary and becomes *convenient*. It is stable, well defined, even chemically defined, and is used, as benzylpenicillin is largely used today, merely as a convenient biological standard. In its third stage, it becomes *superfluous* because chemical assay replaces biological, and the substance, purified, with a guarantee of purity from the chemists, passes over from biology to the highly respectable heaven of the authentic chemical.

Now if you will accept the importance of an early establishment of a standard, we are faced by a contradiction which is the curious result of growing precision of thought about assay developed by biometricians on the one hand, and the rapidly increasing practice of biological assay on the other; namely, that the conditions for a valid assay are precisely those in which biological assay is redundant, or if not redundant, a convenience rather than a necessity. The biometrician allows that our assays are valid only when we can safely assume homogeneity; and by the time we can *prove* homogeneity the standard is in its second age, if not its third, and we do not really need biological assay. Nature apparently likes families of substances; biometricians certainly prefer that active substances should be solitary bachelors. However, in practice this contradiction can be resolved quite simply. In the first place we can assume that however crude and ill-understood our preparations, the requirements of the logical biometrician for valid assay are all fulfilled, and we should refuse to abandon that assumption until we have experimental proof that it is wrong. In the second place, by measuring potency in terms of units (Table IV), we can guard against many of the illogicalities that arise when we hold this compromise position. The unit, arbitrarily defined as the activity of a given weight of a unique preparation

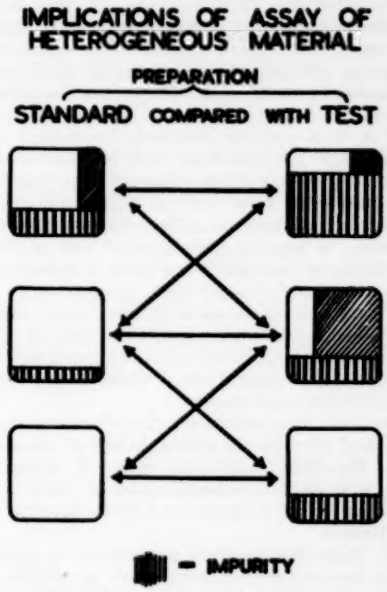


Fig. 3.—Schematic representation of comparison between standard and test preparations of varying degrees of heterogeneity. Unshaded area = active molecular species $\alpha$; cross-shaded area = active molecular species $\beta$; vertically shaded area = inert impurity.

of the substance, is independent of any assumption about purity, homogeneity, or the type of biological system used to reveal specific activity; and it can be used safely in all cases where homogeneity has not been proved. Gram-equivalents are valid when the standard is known to be homogeneous, and the test preparation may be heterogeneous, and the weight basis when both are homogeneous. In some cases, of course, a unit is unnecessary, because the standards are, as it were, single-valued. Thus the sulfarsphenamine, neoarsphenamine, oxophenarsine, and the proposed dimercaprol standards are used as limits of therapeutic activity and toxicity; and therapeutic preparations of the substance are ex-

TABLE IV.—THE MEASURES OF POTENCY APPROPRIATE FOR BIOLOGICAL ASSAY OF MATERIAL OF DIFFERENT DEGREES OF HOMOGENEITY

| Preparations Assayed | | Measure of Potency |
|---|---|---|
| Standard | Test | |
| May be heterogeneous and impure | May be heterogeneous and impure | Unit |
| Homogeneous, may be impure | May be heterogeneous and impure | Unit |
| Homogeneous, pure | May be heterogeneous and impure | Unit or gram-equivalent |
| Homogeneous, pure | Homogeneous, may be impure | Weight |

pected to do no more than exceed the therapeutic potency or be less toxic than the standards by defined proportions.

Generally speaking, the unit is the valid measure of potency wherever a biological test is an *essential* part of the definition of an active substance. It is not possible here to discuss the question of the magnitude of the unit, except to say that where international agreement is reached early in the history of the substance, the magnitude can often be fixed for the convenience of the clinician, the research worker, or the routine assayist. The difficulties in assigning convenient units usually arise because inconvenient units have already been established, and have a degree of priority. Hence, with laboratory or even national standards, laboratory or national units are best fixed with one eye on international adoption. But whatever their magnitude, they should be valid units, i. e., the activity of a given piece of material. The unit is a logical consequence of possible heterogeneity and of the impossibility of completely specifying a biological system. Cat-units, mouse-units, *staphylococcus* units and even man-units can be no more than "range of effective doses in a certain batch of cats, or of mice and men, or of staphylococci" and have no legitimate place in any biological assay where it is possible to use a standard preparation.

From this long argument, then, there emerge some empirical rules for the establishment of standards.

(1) Establish standards early—if desired, as soon as a stable preparation can be made. Even pioneer research is better for a fixed, material point of reference.

(2) Make the standard preparation to satisfy as many known contemporary needs as possible, but do not delay in the hope of better knowledge of the active principles.

(3) Define units of activity only in terms of the weight of the standard preparation; and do not discard units until the standard is a defined chemical.

(4) Do not tie the use of the standard to any particular method of assay, unless substantial heterogeneity is already known.

(5) When substantial heterogeneity is discovered, recognize that any qualification concerning the necessity for a given method of assay imposes a severe restriction on the universality of the standard.

For example, in a memorandum on the newest vitamin D standard, it is necessary to say that "the vitamin D activity for poultry, in international units, of materials that are not known to contain vitamin $D_3$ alone, can be obtained only by assay on poultry". But this necessarily lowers the value of the standard as a universal measure of potency of the D vitamins.

## HIERARCHY OF STANDARDS

Though the International Biological Standards, as they are established and maintained by the World Health Organization, fulfill their purpose as mature, necessary, and convenient standards, there are two obstacles to their being generally useful as early crude standards. The International Standard represents world-wide agreement about the suitability of the standard and about the weight of the standard preparation to which an international unit of potency is assigned. With a rapid exploration and development of new substances, a crude early standard might require changing within a year or less; and though one of the benefits of the early standard is to establish a unit of potency that will have a permanent validity, yet with new knowledge, especially in the recognition of more than one molecular species in the substances, the unit might acquire a changed significance. Something more flexible in conception than the international standard is required, something that does not demand the extensive preliminary research and agreement that precedes the establishment of an international standard. The Expert Committee on Biological Standardization of the W. H. O. also establishes International Reference Preparations, with provisional units of potency for biological substances that are not yet ripe (in the sense, for example, of widespread use in medicine, or widespread interest in research) for promotion to full International Standards. But with these also, the W. H. O., acting for its member states, is responsible for specifying the standard preparation and provisionally assigning unit potency to it. For the even more tentative and perhaps more temporary standard intended to facilitate the early work on antibiotic standards, the Expert Committee on Biological Standardization (14) has recommended setting up a collection of "Author's Preparations" of antibiotics (Table V). The title is important, for it is intended to imply a definitive scientific publication about the antibiotic, designed not only to describe important facts about the substances, but to stimulate work by other scientists. After such a publication, it should be possible, without depriving the author of his rights in harvesting the immediate scientific results of his work, to set aside a number of ampuls containing small samples of the material carrying the antibiotic activity and to assign a provisional unit of potency to a given weight of the material.

TABLE V.—THE HIERARCHY OF INTERNATIONAL STANDARDS

| Standard | Responsibility for Specification | Unit |
|---|---|---|
| "Author's preparation" | Author | Author's (if valid) |
| International Reference preparation | W. H. O. | Provisional unit |
| International Standard | W. H. O. | International unit |
| Pure chemical | ? | Weight |

How the collection should be made is still a matter for debate. Scientific authors might offer specimens of their antibiotics to the W. H. O. when they feel ready to do so; or the W. H. O. might ask for authors' preparations, either on its own initiative or at the request of other workers in the field. Though the Expert Committee on Antibiotics and on Biological Standardization of the W. H. O. might offer suggestions about the standard and the provisional unit, the responsibility for specifying and if necessary modifying the standard would remain with the author, thereby ensuring the flexibility required in any attempt at valid standardization in a shifting

and rapidly developing field. The concept of the author's preparation might well be extended into fields other than antibiotics.

The author's preparation and the Reference Preparation provide models for the early standard. There remains one last point, the problem of the superfluous standard. We have already discarded from the international list androsterone, estrone, and estradiol monobenzoate, as substances now easily characterized by chemical and physical means; and although they are still official standards, logically we should also discard vitamins A and C on the same grounds. Chloramphenicol, again, hardly needs a biological standard. But there are nevertheless biological uses for these substances, and for many other chemicals of biological interest. The Expert Committee on Biological Standardization, and its agents, the National Institute for Medical Research in London and the State Serum Institute in Copenhagen, have sufficient work with *necessary* standards, and a home might be found for these "retired" biological standards in a W. H. O. collection of authentic chemicals which would of course include materials other than these superfluous biological standards, but which might be restricted to substances of agreed biological interest.

In this way, starting with the author's preparation, gradually working through the International Reference Preparation and the International Biological Standards to the Authentic Chemical, there would be available a complete series of reference points that would foster the research and development of substances of biological interest almost from the first report of a new biological effect, to the establishment of the active principle in a textbook.

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# The Reference Standards for Antibiotics*

By HENRY WELCH

UNDER Section 507 of the Federal Food, Drug, and Cosmetic Act, the Federal Security Administrator is given authority to provide for the certification of batches of drugs composed wholly or partly of any kind of penicillin, streptomycin, bacitracin, aureomycin, or chloramphenicol. This authority extends to the establishment of regulations necessary to carry out the provisions and purposes of this section. Among these provisions, the Administrator may prescribe the tests and methods of assay which determine compliance with such regulations.

In Section 146.1 of the regulations on antibiotics the Administrator has defined the antibiotic substances which are subject to certification. For example, in the case of penicillin the definition reads: "Each of the several antibiotic substances (e. g., penicillin F, penicillin G, penicillin X) produced by the growth of *Penicillium notatum* or *Penicillium chrysogenum*, and each of the same substances produced by any other means, is a kind of penicillin." Each of the certifiable antibiotics is defined in a similar manner.

## REFERENCE STANDARDS

**Designation.**—The Administrator has designated the Commissioner of Food and Drugs to establish the master and working standards which are to be utilized to certify each antibiotic. For example, the master standard for streptomycin is specified in Section 146.1(g) as follows: "The term 'streptomycin master standard' means a specific lot of crystalline trihydrochloride calcium chloride salt of streptomycin which is designated by the Commissioner as the standard of comparison in determining the potency of the streptomycin working standard." The streptomycin working standard is designated by this statement: "The term 'streptomycin working standard' means a specific lot of a homogeneous preparation of one or more streptomycin salts."

Each of the antibiotic standards available for distribution by the Food and Drug Administration

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is characterized by the Commissioner of Food and Drugs by describing the standard and its method of storage. The instructions for bacitracin are given as a designation which is typical for all certifiable antibiotic drugs:

> "For the purpose of the regulations published in the Federal Register under Title 21, Part 141 and Part 146, I hereby designate as the bacitracin working standard provided for by section 146.1(n) the bacitracin contained in a number of screw-cap glass jars, each labeled:
>
> BACITRACIN WORKING STANDARD
>
> Potency—52.1 u/mg., when dried 3 hours at 60° C. and a pressure of 5 mm. or less.
>
> The working standard, as described, is at all times stored in the refrigerator under lock and key in Room 6170, South Building, Department of Agriculture, except for brief intervals when portions are removed by laboratory personnel designated by the Director of the Division of Antibiotics."

**Authority.**—The Congress of the United States, in amending the Food, Drug, and Cosmetic Act to provide for the certification of the various antibiotics, delegated the authority for promulgation of regulations to the Federal Security Administrator; the Federal Security Administrator, in turn, has deputed the Commissioner of Food and Drugs to establish standards. It should be noted, however, that under section 146.1(q) the term "Commissioner" refers to the Commissioner of Food and Drugs and to any other officer of the Food and Drug Administration whom he may appoint to act in his behalf for the purposes of the regulations. Under Section 146.2(e), the Commissioner is required to furnish working standards to all interested persons.

**Additional Standards.**—In addition to the standards which have been set up for penicillin, streptomycin, dihydrostreptomicin, aureomycin, bacitracin, and chloramphenicol, it has been necessary to establish standards for tyrothricin, neomycin, and polymyxin as a result of the production of combinations of these drugs with certifiable antibiotics. Thus, bacitracin-tyrothricin troches, streptomycin-bacitracin-polymyxin gauze pads, and neomycin-bacitracin tablets fall into this category. In Table I are listed the reference standards now available for distribution, their potencies and the dates they were designated.

**Distribution.**—Although working standards must be supplied on request to interested persons, the master standards are distributed only rarely, since the main function of the master standards is the establishment of the potency of the working standards. Check assays of the working standard against the master standard are made in the Food and Drug Administration laboratories each month.

**Exemptions.**—Section 507, the amendment which brought about the certification of penicillin and penicillin preparations, was passed by Congress in July, 1945. In March, 1947, streptomycin and streptomycin preparations became certifiable drugs, while in July, 1949, aureomycin, bacitracin, and chloramphenicol and its preparations were added to the list of drugs certified for safety and efficacy by the United States Government. After these three amendments had been passed, reference standards were established for each of the drugs. It should be emphasized at this point, however, that Section 507 requires the Administrator to exempt from the regulations any drug or class of drugs for which, in his judgment, certification is not necessary to ensure safety and efficacy of use. Under this portion of the certification amendment, crystalline sodium and potassium penicillin G were exempted from the regulations in April, 1950.

**First Standard.**—The first reference standard used by the Food and Drug Administration for the assay of penicillin was obtained from the Peoria, Ill., laboratories of the United States Department of

TABLE I.—UNITED STATES FOOD AND DRUG ADMINISTRATION ANTIBIOTIC REFERENCE STANDARDS

| Antibiotic | Standard | Salt | Potency | Date Established |
|---|---|---|---|---|
| Penicillin G | Master | Sodium | 1,667 units/mg. | September 11, 1945 |
| Penicillin G | Working | Sodium | 1,667 units/mg. | February 27, 1948 |
| Streptomycin | Master | $CaCl_2$ complex | 725 μg./mg. | February 27, 1948 |
| Streptomycin | Working | Sulfate | 780 μg./mg. | March 13, 1950 |
| Bacitracin | Master | ..... | 42 units/mg. | October 2, 1950 |
| Bacitracin | Working | ..... | 52.1 units/mg. | February 25, 1952 |
| Dihydrostreptomycin | Master | Sulfate | 800 μg./mg. | October 12, 1949 |
| Dihydrostreptomycin | Working | Sulfate | 800 μg./mg. | October 12, 1949 |
| Aureomycin | Master | Hydrochloride | 1,000 μg./mg. | October 12, 1949 |
| Aureomycin | Working | Hydrochloride | 1,000 μg./mg. | October 12, 1949 |
| Chloramphenicol | Master | ..... | 1,000 μg./mg. | October 12, 1949 |
| Chloramphenicol | Working | ..... | 1,000 μg./mg. | October 12, 1949 |
| Penicillin O | Master | Potassium | O, 100% | January 19, 1951 |
| Penicillin O | Working | Potassium | O, 100% | January 19, 1951 |
| Polymyxin B[a] | Working | Sulfate | 6,300 units/mg. | March 26, 1951 |
| Neomycin[a] | Master | Sulfate | 750 μg./mg. | August 4, 1950 |
| Tyrothrycin[a] | Working | Tyrocidin, 80%<br>Gramicidin, 20% | Tyrothricin, 100% | January 1, 1947 |

[a] Employed as standards in assaying these antibiotics used in combinations with the certifiable antibiotics.

Agriculture in 1943. The potency of this standard was designated as 158 units per mg., but no collaborative assays were made to confirm this value. As soon as crystalline penicillin sodium was made available on a limited scale for laboratory research, it became possible to establish a master standard consisting of the chemically pure sodium salt. Distribution of the crystalline material for routine use as a standard was prohibited by its scarcity. However, this problem was solved by using a secondary standard whose value was determined by assay against the master standard. Three of the major producers of penicillin supplied the Food and Drug Administration with a quantity of crystalline penicillin sodium which was pooled and used as the primary standard. Results of the assays indicated that the value of the crystalline material was approximately 1,600 Oxford units per mg. Eight collaborating laboratories, including six manufacturers, the Food and Drug Administration, and the Northern Regional Research Laboratories, Peoria, Ill., were asked to assay the pooled penicillin sodium standard against the standard in current use in their laboratories. Although there was some variation in the assays reported, it could be seen that an assigned value of 1,650 units/mg. for pure crystalline penicillin sodium would not vary too greatly from its unit value. Therefore this pooled preparation was adopted as the master standard and assigned a potency of 1,650 units/mg.

**Secondary Standard.**—As a secondary standard, a relatively large quantity of penicillin calcium was generously supplied by one of the major manufacturers. The same laboratories which collaborated in establishing a value for the master standard were requested to assay the calcium salt against the master standard, using a value of 1,650 units/mg. Following this collaborative assay, the calcium salt was assigned a value of 370 units/mg. and was accepted as the secondary or working standard. When the regulations for penicillin were promulgated in September, 1945, the penicillin unit was defined as the penicillin activity contained in 0.6 μg. of the master standard, which automatically defined the potency of the penicillin master standard at 1,667 units/mg. The value of 1,667 units was arbitrarily assigned only after members of the industry had been consulted and after consideration had been given to the advantage of rounding off the figure so that each 100,000 units of penicillin would be equivalent to 60 mg. of the master standard. In January, 1946, crystalline sodium penicillin G was established as the new penicillin working standard after collaborative assays in cooperation with the members of the industry had been made.

**Procedure for Establishment of Standards.**—It has been the practice of the Division of Antibiotics of the Food and Drug Administration to follow the same procedure in the establishment of each of the present working and master standards. One or more of the chief manufacturers of the drug is asked to supply a lot suitable for the purpose of making a standard. The laboratories of the Division of Antibiotics study the preparation carefully to determine whether it has the basic characteristics for use as a standard. Each manufacturer interested in the antibiotic is then requested to collaborate in the establishment of the standard potency. At least one of the assay methods used must be that designated by the Division of Antibiotics; a second or third method may be chosen by the manufacturer. The following conditions of assay are assigned: the number of weighings, the number of duplicate assays to be made, and the method of reporting results. After the results are received, an analysis is made of the values, and the potency of the standard is established on the basis of this data. When an agreement has been reached between the industry and the Food and Drug Administration, the preparation is designated as the standard by the Commissioner of Food and Drugs.

# Reference Standards for Biological Products*

By WILLIAM G. WORKMAN

As far as the United States is concerned, the first problem of standardization of a therapeutic serum may be said to have had its beginning in September, 1894, when Dr. William H. Park of the Bureau of Laboratories of the City of New York began immunization of the first horse in this country to produce diphtheria antitoxin. After immunization, serum was prepared from the horse and used to treat cases of diphtheria in January, 1895.

Although satisfactory methods for the potency testing of diphtheria antitoxin and tetanus antitoxin were being developed principally by Ehrlich and von Behring in Germany, authorization for

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official standardization in this country did not come until the enactment of Public Law 244, commonly known as the "Biologics Law," which became effective on July 1, 1902. In essence, this law provides that certain specified biological products may be prepared only by inspected and licensed laboratories and that appropriate regulations must be promulgated to control the safety, purity, and potency of such products.

The need for the control of their safety was emphasized in 1900 by the death from tetanus of a number of children in St. Louis who had been ill with diphtheria and were treated with antidiphtheritic serum from a horse later shown to have been in the incubation stage of tetanus with tetanus toxin in the blood at the time of bleeding.

That a real need existed for a uniform standard of potency is illustrated by tests of tetanus antitoxins made at the Hygienic Laboratory while the United States unit was being developed, but before it was adopted. One sample from the New York State Department of Health at Albany, N. Y., was labeled 0.5 unit per cubic centimeter but was found to contain 434 units; another was labeled 0.75 unit but contained 769 units. A sample labeled 700 units from the New York City Department of Health was found to contain 166 units. A product of the H. K. Mulford Company was labeled 100,000 units, but contained only 77 units; another labeled 6,000,000 units contained 90 units. A Parke, Davis and Co. sample was labeled 60,000 units; when tested, it contained 700 units. Samples from France and Italy for which the unit value was not stated were discovered to contain 40, 40, 83, 66, and less than 50 units, respectively. Apparently each manufacturer had devised his own unit or means of estimation, and had no common basis with other manufacturers for expressing potency. Moreover, in actual potency, the antitoxins varied from only fairly good to very poor.

Reliable methods of testing and standardization, which are now taken for granted, were slow in development. French investigators attempted to evaluate diphtheria antitoxin by determining the amount needed to protect against an infecting dose of virulent organisms. Protection was shown but the estimated potency values were irregular and inconsistent. In both Germany and America, attempts were made to estimate potency by determining the combining value of the antitoxin with a given amount of a so-called "standard" toxin. In fact, the present diphtheria antitoxin unit originated as the amount which would supposedly neutralize 100 MLD of toxin. It is now well known that the method is in error because the combining value of crude toxins does not bear a constant ratio to their toxicity. An important step forward was taken when Ehrlich devised the indirect comparison of the unknown antitoxin with a standard antitoxin by combining each with a test dose of toxin.

## GENERAL PRINCIPLES

With this background it seems appropriate to attempt to summarize the present basis of methods of biological standardization. Although many factors, some of which are not fully understood, may influence a biological reaction, these factors can usually be controlled so that a result sufficiently accurate to be valid and useful can be obtained. Some of the principles which should be kept in mind in the design and interpretation of biological tests are as follows:

(1) The unknown substance should be tested in comparison with a fixed amount of a stable physical standard.

(2) The standard preparation must be capable of producing a constant response in the test animal.

(3) The unknown substance being tested must be capable of producing the same response as the standard preparation.

(4) The unknown and the standard preparations must be either pure substances (which is practically never the case), or they must not contain impurities or added substances which modify the action of the active principle.

(5) It must be possible to obtain a significantly different response with graded doses of the substances tested.

**Physical Standards.**—As stated in (1), the physical standard must be stable. Various means are used to attain stability. The United States diphtheria and tetanus antitoxin standards are sterile preparations, dried and sealed under vacuum in amber glass U-tubes with phosphorus pentoxide in the opposite arm of each tube. The standards are kept under refrigeration; any tubes showing absorption of moisture because of cracks or defective sealing are discarded. Periodically, a tube of the dried standard is dissolved and diluted in 50% glycerin for issue as the working standard. Sometimes substandards are prepared for laboratory use, but the manufacturer's final potency test of a lot of antitoxin is always made against the official standard.

Although it might be desirable to re-establish an antitoxin standard *de novo* from some natural phenomenon, as in the case of the standard meter by comparison with the wave length of light, no such method has been devised for an antitoxin standard. When it becomes necessary to prepare a new biological standard, its potency is determined by careful comparisons against existing standards. The question has been raised as to whether a standard may not gradually deteriorate and thus result in a smaller value of the official unit. When a new working standard is made up from a portion of the standard preparation, it is always compared with the previous working standard. In addition, occasional comparisons are made with the international and other standards. The probability of all the standards falling off at the same rate seems very remote indeed.

The assumption of a constant response in (2) and (3) raises the question not only of homogeneity of the substance being tested, but also of uniformity of the test animal. It is sometimes said that, since the test animal is never uniform, the results of a biological test can never be valid. It should be remembered that even such physical measurements as those of dimension and weight are never absolutely accurate; it is required only that the error be within such limits so that it can be disregarded for a specific purpose. From the same viewpoint, it should be required of a biological test only that it gives a result of sufficient accuracy to be valid for a given purpose.

**Selection of Test Animal.**—Much attention has been given to the selection of the test animal. Frequently selection may be made on the basis of species, strain, sex, age, weight, diet, and other factors to improve the reliability of the results. It is sometimes possible, as in the case of insulin, to give repeated doses in the same animal, or, in the case of the intradermal testing of diphtheria antitoxin or of tuberculin, to give simultaneous injections in the same animal at many sites. The test animal is only one of the many possible variables in a biologic test; depending upon the nature of the test being done, the variability of the animal may be of relatively minor importance. In the diphtheria and the tetanus antitoxin potency test, the animal serves only as an indicator of whether neutralization has occurred in the *in vitro* mixture of toxin and antitoxin.

From a practical viewpoint in the control of biologic products, it should be pointed out that the necessity for preciseness of potency determinations varies with the nature and clinical application of the individual product. In the case of insulin, it is essential that the potency of the product be known with a fairly high degree of accuracy, since either an overdose or an underdose may give rise to serious difficulties. No upper limit has been placed on the immunizing potency of preparations used for prophylactic immunization, and overdosage is not known to be harmful except when the sensitivity of the individual may be a factor. No damage is known to result from the administration of any amount, however large, of a therapeutic antitoxin as far as the specific antibody content is concerned. From the clinical viewpoint, it is only necessary in most cases to have reliable assurance of an adequate minimum potency of the product. However, precise assays are frequently a valuable guide in manufacturing procedures and in research investigations.

**Standards Other Than Physical.**—A discussion of reference standards for biological products would not be complete without mention of the fact that physical standards of reference have not been prepared for a number of products. Some of these products have been used extensively for many years.

In some cases, it has seemed difficult or impossible to prepare a physical standard; in others, the need for such a standard has not been so urgent.

Some assurance of potency, although with less certainty, may be provided by means other than a physical standard.

Manufacturing procedures which should result in a good product may be specified. For example, in the preparation of typhoid vaccine, specifications may include a strain of the organisms of known antigenicity, the culture medium, the conditions of cultivation and harvesting, the bacterial count, and the preservative. However, there is hardly any method of preparing typhoid vaccine or any other product which does not result occasionally in a product lot of unaccountably low potency, as all factors which may affect potency can seldom be controlled. Some kind of potency test of each lot of the final product is almost a prime necessity.

With some products, it is possible to test for potency in animals without using a standard, as in the case of tetanus toxoid. Such control seems adequate for tetanus toxoid. There were so few cases of tetanus in the U. S. military forces during World War II that no question of the potency of the toxoid arose. Each lot was tested in a group of animals, which minimized the variable individual animal response; in addition, each lot was tested both by the manufacturer and by the Laboratory of Biologics Control.

Nevertheless, physical standards are desirable and the trend both in the international field and in this country is toward developing such standards as rapidly as possible for all products which must be tested for potency.

## INTERNATIONAL STANDARDS

Although technically the official physical standards in the United States provided by law and regulations are those established by the National Institutes of Health, there are compelling reasons why biological standards should be uniform throughout the world. This may best be accomplished through international cooperation.

International standards are recognized in this country by making the official United States standard equivalent to the international standard so that our standard becomes, in effect, a substance of the international standard. In cases where an international standard was established first, our own was made equal to it. In the few instances in which the United States standard is older, the international standard was made equal to ours. In several instances, it has been possible to share the material so that there could be no question of equivalence.

With the exception of the tetanus unit, there has never been any disagreement in value between the international standards and our own. In 1950, the international tetanus unit was re-established at twice its former value so that the United States unit and the international unit are now the same.

The Public Health Service and the people of this country who are concerned with the manufacture and the use of biological products in clinical medicine are deeply grateful for the excellent work being done in the field of international standardization. This service may not be the most spectacular activity of the World Health Organization but it is a truly valuable contribution to international understanding and good will.

# A Proposed Method for Determining the Effect of Deodorants on the Elimination of Alcohol Breath*

By Z. F. AHMED, J. A. CAMPBELL, J. E. CHRISTIAN, and J. R. EATON

**A preliminary *in vitro* study to determine the effect of alcohol vapor on the surface tension of water was carried out. A similar determination of the effect of alcohol breath was tried with positive results, which suggested a method for determining the effect of deodorants on the elimination of alcohol breath.**

ALCOHOL lowers the surface tension of water considerably when mixed with it (1, 2). The great affinity of alcohol and its vapors (3) toward water will bring about drastic changes in the surface tension of water in a rather short time. In the following experiments, attempts were made to correlate the various dilutions of alcohol with the effects of their respective vapors on the surface tension of a pendant drop of water by using different alcohol-water mixtures.

The fact that high dilutions of alcohol brought about a perceptible change in the surface tension of water encouraged us to try the effect of alcohol breath on the lowering of surface tension. In this case an even more perceptible change occurred, possibly due to the higher oxidation level of the metabolic products which expectedly would be more odoriferous.

The positive results obtained stimulated the proposal of a rather simple technique to determine the effect of chlorophyll and derivatives on the elimination of alcohol breath of humans when taken internally. The instrument used was that described by Tanyolac and Eaton (4), and modified by Campbell, *et al.* (5). The technique used was based upon work reported by the authors of this paper (5).

## EXPERIMENTAL

**Preliminary Experiments.**—The following experiment was carried out to determine the general effect of alcohol on the surface tension of water.

A quantity of 5 cc. of ethyl alcohol, U. S. P. was diluted with 5 cc. of water and then introduced into the chamber (3 x 3 x 3 in.). Exactly 1 cc. of water at 28° was used as the testing liquid. The results obtained are shown in Table I.

In another experiment, using 10 cc. of 2.5% v/v alcohol as odorant, the drop of water fell instantaneously after the surface tension attained 49 dynes $cm^{-1}$. This was considered the critical value below which the water drop was not stable.

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TABLE I.—EFFECT OF ALCOHOL ON THE SURFACE TENSION OF WATER

| Time after Alcohol Introduced, Sec. | Surface Tension, Dynes $Cm^{-1}$ |
|---|---|
| 0 | 71.6[a] |
| 45 | 56.5 |
| 60 | 53.2 |
| 75 | 51.7 |
| 90 | 50.7 |
| 105 | Drop fell spontaneously while taking the picture. |

[a] This value remained constant before introduction of the odorant.

**Determination of the Effect of Various Dilutions of Alcohol on the Surface Tension of Water.**—Aliquots of 10 cc. of each of the following dilutions (v/v) of alcohol were introduced separately into the chamber as odorants. Exactly 1 cc. of water at 28° was used as a testing liquid in each case: 100, 90, 80, 70, 60, 50, 40, 30, and 20%.

The results shown in Fig. 1 indicate that the higher the concentration of alcohol, the greater the depression in the surface tension of water and the shorter the time taken by the water drop to become unstable and fall.

The fairly constant increments between the curves of various alcohol dilutions affords a possible correlation between the alcohol concentrations and their effect on the surface tension of water.

The dotted lines Fig. 1, are extrapolations to the theoretical line (49 dynes $cm^{-1}$) at which point the drop of water is unstable. During the actual experiment, the drop fell between the time the last picture was taken and the next time interval.

**Determination of the Effect of Higher Dilutions of Alcohol on the Surface Tension of Water.**—Aliquots of 10 cc. of each of the following dilutions

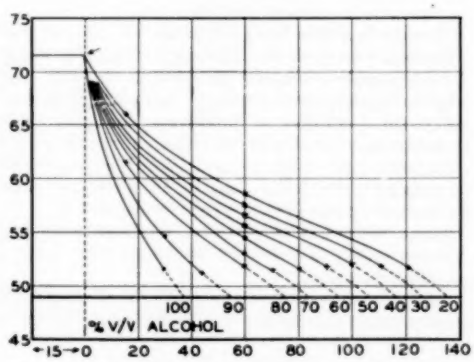


Fig. 1.—The effect of lower dilutions of alcohol on the surface tension of water.

(v/v) of alcohol, U. S. P. were introduced separately into the chamber as odorants. Exactly 1 cc. of water at 28° was used as a testing liquid in each case: 10, 5, and 1%.

The results shown in Fig. 2 indicate that the lower the concentration of alcohol, the less the depression of the surface tension of the water. At the lower concentrations, the drop remained stable and did not fall.

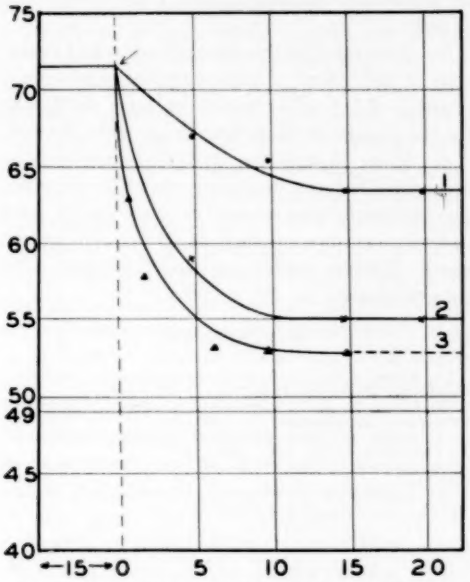


Fig. 2.—The effect of higher dilutions of alcohol on the surface tension of water. *1*—1% (v/v) alcohol. *2*—5% (v/v) alcohol. *3*—10% (v/v) alcohol.

**The Effect of the Alcohol Breath and in Vitro Deodorized Alcohol Breath on the Surface Tension of Water.**—Ten cubic centimeters of U. S. P. alcohol was taken internally. Fifteen minutes later, 10,000 cc. of breath was bubbled through 20 cc. of water which was then introduced as odorant into the chamber containing a stable pendant drop of water at 28°.

The technique used for collecting the odor in the breath was as follows (Fig. 3): A small flask was fitted with a 2-hole rubber stopper. Two glass tubes were fitted in the stopper, one of them extending beneath the surface of 20 cc. of water. The shorter tube was attached by rubber tubing to an inverted 5-gal. carboy filled with water. The carboy was graduated to enable measurement of the amount of water displaced. The subject bubbled breath through the water until 10,000 cc. of water was displaced. The 20 cc. of water was put in a standard sized tin cup and immediately placed in the measurement chamber which contained a pendant drop of water at 28°. The effect upon the surface tension of the pendant drop, produced by the dissolved odors in the water, was noted at various time intervals after introduction of the sample into the chamber.

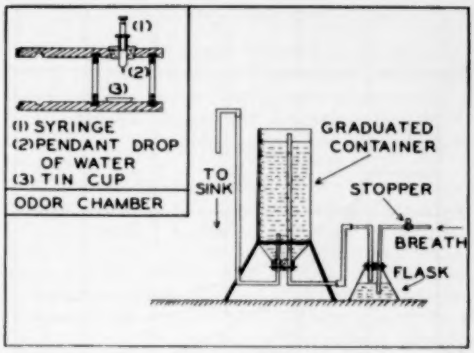


Fig. 3.—Apparatus for collecting a sample of breath.

Another experiment was carried out on a second day by the same individual in exactly the same way but with the addition of 3 mg. of copper-fixed chlorophyllins to the 20 cc. of water before bubbling the breath through the system. Attention was paid to maintain a constant slow bubbling of the breath through the water.

The results shown in Table II indicate that the alcoholic breath as measured depressed the surface tension of water to a perceptible degree and that the use of chlorophyllins *in vitro* had a definite deodorizing effect.

TABLE II.—THE EFFECT OF ALCOHOL BREATH AND CHLOROPHYLLIN-TREATED ALCOHOL BREATH ON THE SURFACE TENSION OF WATER

| After Introduction of the Odorant, Time, Min. | Surface Tension of Water, Dynes $Cm^{-1}$ | |
|---|---|---|
| | Untreated Alcohol Breath | Chlorophyllin-Treated Alcohol Breath |
| 0 | 71.6* | 71.6* |
| 25 | 65.5 | 69.5 |
| 35 | 61.0 | 67.5 |
| 45 | 60.0 | 64.0 |
| 55 | 60.0 | 64.0 |

* These values remained constant over a period of time prior to the introduction of the odorant.

**In Vivo Study of the Effect of Chlorophyllins on the Alcohol Breath.**—A sample of breath was taken as previously described from three normal individuals with no sign of halitosis and was tested for any effect on the surface tension of water. No effects were noted.

The same individuals, free from detectable breath contaminants, were given 30 cc. of Bourbon whiskey (bonded, 100 proof). After rinsing the mouth thoroughly with water, each subject took a tablet containing 40 mg. of chlorophyllins. Thirty minutes later a breath sample was taken and was placed in the chamber. Photographs of the drop were taken every five minutes for a period of forty-five minutes.

Twenty-four hours later the same experiment was repeated on the same individuals under exactly the same conditions without the administration of the chlorophyllins.

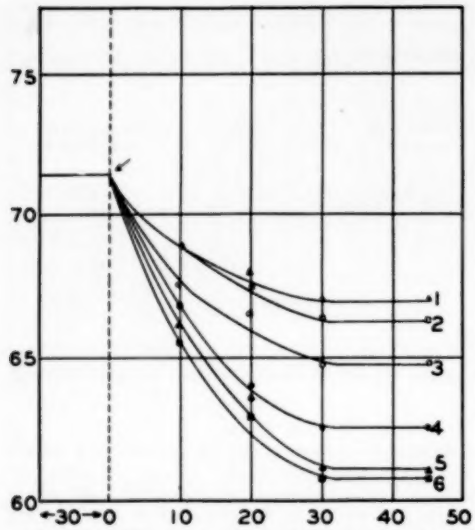


Fig. 4.—Effect of chlorophyllins on alcohol breath. *1*—Subject A with chlorophyllins administered. *2*—Subject B with chlorophyllins administered. *3*—Subject C with chlorophyllins administered. *4*—Subject C without chlorophyllins. *5*—Subject A without chlorophyllins. *6*—Subject B without chlorophyllins.

The results shown in Fig. 4 indicate that the magnitude of depression in the surface tension of water caused by alcohol breath varied with the individual, but the chlorophyllins produced a measure of deodorization of alcohol breath in each case.

## SUMMARY

1. A correlation between the different alcoholic dilutions and the effect of their respective vapors on the surface tension of water exists.

2. Chlorophyllins exert a deodorizing action on the human alcoholic breath both *in vitro* and *in vivo*.

3. A method for the determination and evalution of the effect of chlorophyllins, and consequently of any other substance, as a deodorant for the human alcoholic breath has been devised.

4. Even though the individual response to chlorophyllins as a deodorant varied to some degree, chlorophyllins reduced alcoholic breath odor in every instance (as high as 60 per cent in one case). Further studies are needed for more critical evaluation.

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# A Study in the Measurements of Odors and the Deodorizing Effects of Certain Materials*

By J. A. CAMPBELL, Z. F. AHMED, J. E. CHRISTIAN, and J. R. EATON

**The effects of air contaminations caused by certain standard odorants on the surface tension of a liquid drop were determined with and without the presence of deodorants. An attempt to develop a standardized statistically sound technique for measuring the effect of deodorants was made.**

A VITAL need in the science of olfaction is a truly scientific and reliable method of measuring odor intensity. A number of ingenious devices have been constructed and utilized with varying degrees of success. The one great weakness present in most of the comprehensive systems is that they depend upon that extremely variable factor, human perception. However delicate and sensitive the human nose may be, too many variable factors (1–3) make it extremely difficult to standardize accurate odor measurement. The thresholds of odor perception (2, 3) vary widely with many factors. Thresholds extend from the very keen to that of total anosmia. Anosmia can be permanent or temporary, and the temporary status may vary from day to day depending on conditions such as colds and catarrh of the nose. It is fairly evident that

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thresholds can vary with many other factors such as age, sex, fatigue, personal habits, environment, and psychological make-up of the individual. If we might compare the sense of smell with the other senses, such as sight and hearing, we would logically expect a gradual decline in acuity of perception as a person grows older. It is well recognized that people vary in susceptibility to suggestion (4), which presents another problem in odor perception measurements.

Of all the known devices constructed for the purpose of odor intensity measurement, that designed by Tanyolac and Eaton (5) appears to be the most promising. Their technique, the only one in which the factor of human judgment in odor perception is entirely eliminated, was developed as a result of observations (6) that certain phenomena exist in relation to contaminants present in the air, prominent among which is change in surface tension of liquids when in the presence of contaminated air. In the experiments of Tanyolac and Eaton, it was assumed that odors were forms of air contaminants, and the device used was constructed to utilize the pendant drop method for measuring surface tension change. The drop was suspended in a metal chamber to prevent contaminants other than the odor to be tested from coming in contact with the liquid. It was observed that when certain odorous substances were placed in the chamber, there was a characteristic change in surface tension of the liquid which increased with the surface area exposed and also with the decrease in volume of the chamber. Examples of the odorants used were camphor, cloves, and rubber; and examples of the detecting liquids were distilled water, mercury, and mineral oil.

In view of the potentialities of the technique described, the objective of the present research was to develop some practical applications in the field of odor measurement.

A subject of current interest is the efficacy of certain materials used for deodorant purposes, such as chlorophyll and its derivatives. In this work, methods were devised whereby the ability of any substance to reduce the intensity of an odor could be determined. Several commercial samples of chlorophyll and its derivatives were selected for these tests.

Due to the fact that the exact concentration of active principles in some of the samples was unknown, a comparison of efficiency on an exact weight basis was not attempted at this time. The chief objective was to determine the answer to the controversial question of whether or not each sample possessed some measure of deodorizing ability.

## EXPERIMENTAL

The device used in the following study was principally that described by Tanyolac and Eaton (5). Certain improvements (Fig. 1) such as the container for the odorous material and the syringe for forming the pendant drop were added and proved to be beneficial.

The effect of a measured quantity of a standard odorous material, e. g., diallyl sulfide ($CH_2=CH—CH_2)_2S$, cineole ($C_{10}H_{18}O$), and eugenol ($C_6H_3.C_3H_5.OCH_3.OH$) on the surface tension of a testing liquid was determined in the device. The same experiment was then repeated after shaking the same amount of the odorant with different quantities of the deodorant for a certain period of time at a certain temperature. The amount of the deodorant that can fix a certain amount of the standard odor (as shown by the constancy of the surface tension of the testing liquid) could be calculated.

A study was made by using different deodorants under identical conditions. A control experiment using the deodorant alone was carried out previously.

The details of the technique are described in the following experiments.

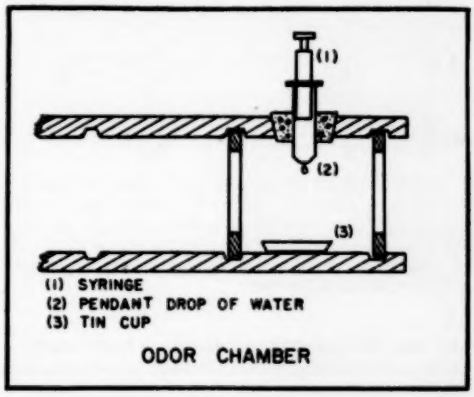


Figure 1.

**The Deodorizing Effect of Chlorophyllins on Eugenol.**—Five cubic centimeters of a saturated aqueous solution of eugenol were shaken mechanically in a well-stoppered 5-ml. volumetric flask for fifteen minutes at room temperature (25°) and then transferred completely with the aid of 5 cc. of distilled water into a tin weighing cup (6 cm. in diameter).

The cup was then introduced into the chamber (3 x 3 x 3 in.) of the instrument where a stable drop of medicinally pure water was pending from a 1-cc. tuberculin syringe with a previously calibrated nozzle.

The surface tension determination of the pure water was carried out for a period of at least fifteen minutes before the introduction of the odorous material and sixty minutes afterward. Photographs were taken at five-minute intervals.

The same experiment was then repeated in exactly the same way after shaking 5-cc. portions of the saturated aqueous solution of eugenol separately with 0.1, 0.5, 0.75, and 1.0 Gm. of chlorophyllins (commercial sample 1) for a period of fifteen minutes.

The results shown in Fig. 2 indicate that 5 cc. of a saturated aqueous solution of eugenol produced a marked depression on the surface tension of water

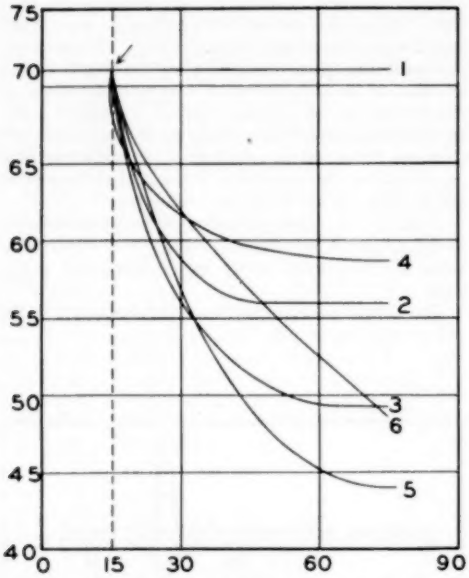


Fig. 2.—Effect of deodorants on eugenol solution. *1*—1.0 Gm. and 0.75 Gm. of chlorophyllins, Sample 1. *2*—0.5 Gm. of chlorophyllins, Sample 1. *3*.—0.1 Gm. of chlorophyllins, Sample 1. *4*—0.1 Gm. of charcoal. *5*—0.1 Gm. of talc. *6*.—Saturated solution of eugenol alone.

and that the use of chlorophyllins reduced the magnitude of this depression through its deodorizing effect.

When 0.75 Gm. and 1.0 Gm. of chlorophyllins were used, no depression in the surface tension of water was noticed. This indicated that all the eugenol was deodorized, which fact was also confirmed organoleptically.

When 0.5 Gm. of chlorophyllins was used, a marked but incomplete deodorizing effect was obtained. When 0.1 Gm. was used, the effect was less pronounced.

The control experiment, in which a heavy suspension of chlorophyllins in water was used as a source of contamination, showed no effect on the surface tension of water.

**Testing the Surface Tension Technique for Reliability and Confidence.**—Five experiments, using a saturated aqueous solution of eugenol as odorant, were carried out under exactly the same conditions (box size, 3 x 3 x 3 in.; temperature, 25°) on different days. The results obtained are shown in Table I.

**The Deodorizing Effect of Chlorophyllins on Eugenol Compared to the Effects of Other Materials.**—Quantities of 0.1 Gm. of each of the following substances was used separately as a deodorant for 5 cc. of a saturated aqueous solution of eugenol: chlorophyllins (commercial sample 1), vegetable charcoal (Merck), and purified talc (U. S. P.). Using exactly the same technique as described before, each of these substances was shaken with the eugenol solution for fifteen minutes prior to their introduction into the chamber.

The results shown in Fig. 2 indicate that both the chlorophyllins and charcoal demonstrate considerable deodorizing ability by this technique. Talc, on the other hand, shows no deodorizing effect whatsoever, but seems to enhance the odor.

In a separate set of experiments, 5-cc. quantities of the saturated solution of eugenol were shaken for sixty minutes alone, and with 0.1 Gm. of chlorophyllins, 0.5 Gm. of chlorophyllins, and 0.1 Gm. of charcoal in an attempt to determine the effect of time of contact on deodorization.

The results shown in Fig. 3 indicate that the deodorizing effect was more pronounced with sixty minutes than with fifteen minutes of shaking; with the eugenol alone, there was also a slight difference.

**Determination of the Deodorizing Effects of Various Commerical Samples of Chlorophyll and Its Derivatives on Eugenol, Cineole, and Allyl Sulfide.**—Each of the following commercial products was used separately as a deodorant for 5 cc. of a saturated

TABLE I.—LOWERING OF THE SURFACE TENSION OF WATER IN DYNES $CM^{-1}$ PRODUCED BY A SATURATED SOLUTION OF EUGENOL

| Time, Min. | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 | Expt. 5 | Mean | Standard Deviation of the Mean[a] |
|---|---|---|---|---|---|---|---|
| 0 | 71.6 | 71.6 | 71.6 | 71.5 | 71.5 | 71.6 | 0.03 |
| 15 | 71.6 | 71.6 | 71.6 | 71.5 | 71.5 | 71.6 | 0.03 |
| 15 | Odorant Introduced | | | | | | |
| 25 | 63.2 | 65.8 | 65.9 | 65.5 | 65.5 | 65.2 | 0.50 |
| 35 | 61.5 | 60.5 | 60.3 | 60.0 | 60.0 | 60.5 | 0.31 |
| 45 | 57.1 | 58.2 | 57.0 | 56.8 | 56.0 | 57.0 | 0.36 |
| 55 | 53.4 | 53.5 | 52.8 | 53.3 | 53.8 | 53.3 | 0.16 |
| 65 | 52.8 | 52.5 | 51.8 | 51.8 | 51.8 | 52.1 | 0.21 |
| 75 | 48.9 | 48.6 | 48.2 | 48.7 | 48.5 | 48.6 | 0.11 |

[a] $M = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$ where: $M$ = standard deviation of the mean; $d$ = difference of observation from arithmetic average of a number of observations; $N$ = total number of observations.

The statistical treatment of the data obtained in this series of experiments demonstrates the high degree of reliability which can be expected from this technique.

aqueous solution of eugenol, cineole, or allyl sulfide: Sample No. 1, chlorophyllins in powder form; Sample No. 2, chlorophyllins in powder form; Sample No. 3, chlorophyllins in powder form; Sample No. 4, chlorophyllins in aqueous suspension; Sample No. 5, coppered chlorophyllins in powder form; Sample No. 6, chlorophyll (oil soluble) in viscous liquid. All of the six commercial samples studied were characterized by spectrophotometric tests. A spectrophotometric curve was determined for each of the samples in the visible spectrum, using the Beckman DU spectrophotometer. All of the samples produced the same general type of curve. Each showed two significant absorption bands, the first ranging from 400 to 410 mμ, and the second from 615 to 660 mμ. The findings are in agreement with other published results (7) which indicate that all the samples tested contained chlorophyll or its derivatives.

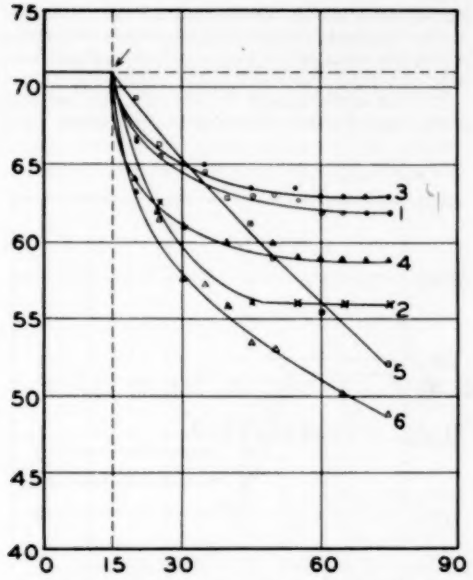


Fig. 3.—Effect of time on deodorizing of eugenol solution. *1*—0.5 Gm. of chlorophyllins for sixty minutes. *2*—0.5 Gm. of chlorophyllins for fifteen minutes. *3*—0.1 Gm. of charcoal for sixty minutes. 4—0.1 Gm. of charcoal for fifteen minutes. *5*—Eugenol solution alone for sixty minutes. *6*—Eugenol solution alone for fifteen minutes.

Using exactly the same technique as described before, 0.5 Gm. of each of these samples was shaken separately with the odorant solution before being placed in the chamber.

The results shown in Figs. 4, 5, and 6 indicate that each sample produced some degree of deodorization upon the odorant solutions tested.

Although each of the chlorophyll derivatives showed a degree of deodorization with each of the odorants used, the relative magnitudes of their effectiveness differed from one odor to the next, indicating some specificity as far as deodorizing properties are concerned.

The effect of a saturated aqueous solution of cineole on the surface tension of water is quite perceptible, being greater than that of eugenol or allyl sulfide. The relatively wider range of differences between the effects of various samples, as well as the ease of decontamination, made the choice of cineole as a testing odorant advantageous.

The effect of a saturated aqueous solution of allyl sulfide alone on the surface tension of water is less than that of both eugenol and cineole individually.

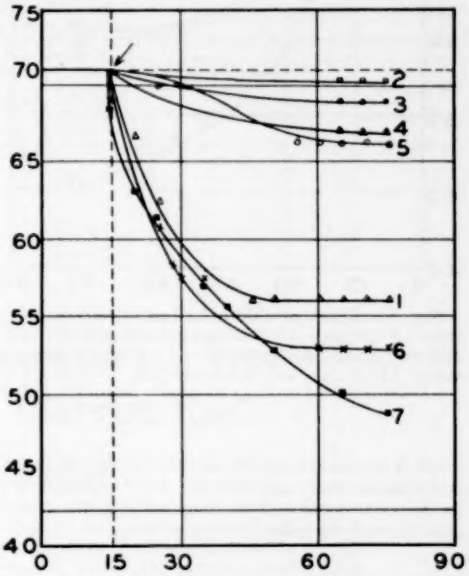


Fig. 4.—Effect of chlorophylls on eugenol solution. *1* through *6*—Commercial chlorophyll samples numbered consequently. *7*—Eugenol solution.

The difficulty of decontamination, as well as the narrow range of differences between the effect of various samples, made allyl sulfide unsuitable as an odorant.

**Determination of the Effect of Eugenol and Chlorophyll-treated Eugenol on the Surface Tension of Mercury.**—In this set of experiments, water-stabilized mercury was used as a testing liquid instead of water.

Five cubic centimeters of a saturated aqueous solution of eugenol was shaken at 28° for fifteen minutes alone and separately with 0.5 Gm. each of commercial samples 1, 5, and 6.

In each case, the tin cup containing the odorant was introduced into the chamber (3 x 3 x 3 in.) of the device where a water-stabilized drop of mercury was pending from an 8-mm. capillary tube firmly connected to a 1-cc. syringe by a piece of plastic tubing. The surface tension determination of the water-stabilized mercury was carried out for a period of at least thirty minutes before the introduction of the odor and sixty minutes thereafter. Photographs were taken at ten-minute intervals.

The results shown in Fig. 7 indicate that 5 cc. of a saturated aqueous solution of eugenol produced a

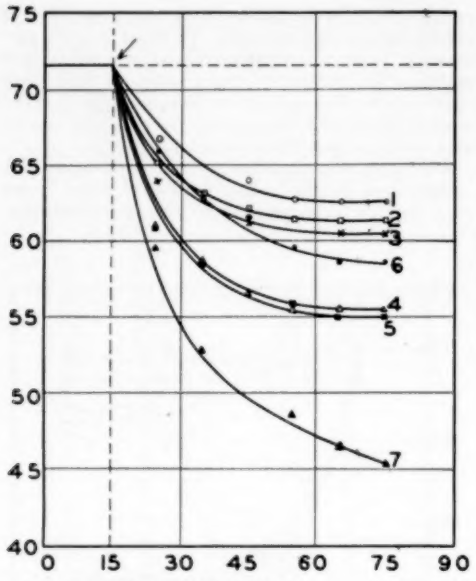


Fig. 5.—Effect of chlorophyllins on cineole solution. *1* through *6*—Commercial chlorophyll samples numbered consecutively. *7*—Eugenol solution alone.

marked depression on the surface tension of water-stabilized mercury and that the use of chlorophyll or its derivatives reduced the magnitude of this depression through their deodorizing effect.

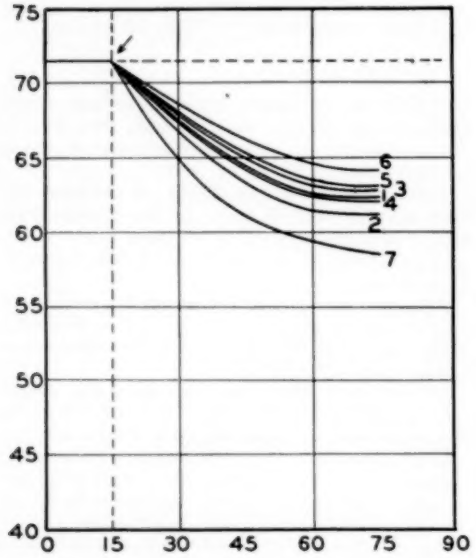


Fig. 6.—Effect of chlorophylls on allyl sulfide solution. *1* through *6*—Commercial chlorophyll samples numbered consecutively. *7*—Allyl sulfide solution alone.

## DISCUSSION

Of materials investigated, water and water-stabilized mercury have proved to be the best testing liquids. Both have a reasonably high surface tension which is constant for a relatively long period of time.

Attempts to find other testing liquids which could qualify for use instead of water and mercury were unsuccessful. It was found that salt solutions tended to show a rapid fall in surface tension with time. Most of the pure organic and inorganic liquids reported in the literature have either low surface tensions, which limits the amount of lowering which can occur, or they are available in liquid form only at temperatures well above room temperature.

The use of the syringe to provide a pendant drop in the case of water proved to be convenient. The

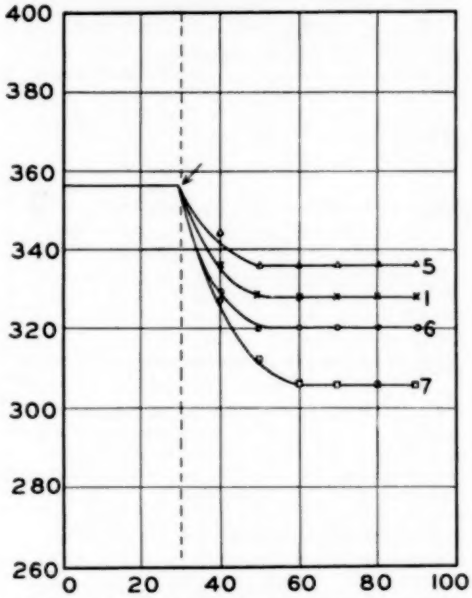


Fig. 7.—Effect of chlorophyll-treated eugenol on the surface tension of mercury. *1*—Commercial chlorophyll sample 1. *5*—Commercial chlorophyll sample 5. *6*—Commercial chlorophyll sample 6. *7*—Eugenol solution alone.

formation and control of the size of the drop was very easy and prompt. The amount of water in the syringe could be maintained at any particular level, eliminating, to a considerable extent, the variance in the surface tension due to internal forces.

The use of tin cups to hold the odorant provided a good means of having a light dispensable container which, while impermeable to liquids, allowed for a larger exposed surface and a constant geometrical position in the odor chamber.

As far as the effect of time on deodorization was concerned, it was found that although a longer time might bring about a greater lowering of surface tension, the choice of fifteen minutes of shaking was found more convenient.

In all the experiments, the results were matched by organoleptic tests. In every case where there was an odor, there was a change in the surface tension; where there was no change in the surface tension, there was no odor perceptible. However, in cases of very faint odors, which were hardly perceptible by smell, there was a noticeable change in the surface tension of the testing liquid.

## SUMMARY

1. A technique for quantitatively measuring the effect of deodorants has been described.

2. The effects of air contaminations caused by certain standard odorants, namely, eugenol, cineole and allyl sulfide, on the surface tension of water as well as the deodorizing effect of certain commercial chlorophylls and chlorophyllins on these odorants were determined by applying the proposed technique.

3. The validity of the technique was ascertained by testing it for reliability and confidence.

4. The technique may be used with accuracy to measure quantitatively the relative strengths of odor.

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# Effects of Aspirin, Aminopyrine, and HPC upon Glycosuria in the Diabetic Rat*

By DWIGHT J. INGLE†

**Aspirin, aminopyrine, and HPC (3-hydroxy-2-phenylcinchoninic acid) were each tested in partially depancreatized male rats which were force-fed a medium carbohydrate diet. Following a control period of several weeks, the rats received a drug by subcutaneous injection in daily amounts of 40, 80, and 160 mg. for seven days at each dose level. There was a marked suppression of glycosuria which was proportional to the dose of aspirin. Aminopyrine had no significant effect in severely diabetic rats but did cause a slight temporary suppression of glycosuria in animals with less severe diabetes. HPC had no significant effect upon the level of urinary glucose, but some of the rats died during the administration of 160 mg. daily. The failure of these drugs to cause exacerbation of the diabetic state is evidence against the hypothesis that they are capable of inducing a state of hypercorticalism in the rat.**

THE pharmacologic properties of aspirin, aminopyrine, and HPC (3-hydroxy-2-phenylcinchoninic acid) are similar. Each drug is capable of stimulating the anterior pituitary to activate the adrenal cortex via the increased release of corticotropin (1). It has been postulated that the pharmacologic and therapeutic effects of these drugs may be mediated by an increased secretion of adrenal cortical steroids (2). If these drugs are capable of causing a state of hypercorticalism, they should each cause exacerbation of diabetes in the rat, since each of the 11-oxygenated steroids of the adrenal cortex is diabetogenic in this animal (3).

In these experiments it was found that aspirin caused amelioration of the diabetic state, aminopyrine caused a temporary suppression of glycosuria in the mildly diabetic rat, and HPC was without any significant effect upon the level of urinary glucose.

## METHODS

Infection-free male rats of the Sprague-Dawley strain were partially depancreatized at a weight of 275 Gm. by the procedure described by Ingle and Griffith (4). The experiments were conducted in an air-conditioned laboratory with the temperature maintained at 74–78° F. and the humidity at approximately 50% of saturation. After the animals had recovered from the operation and had reached a weight of approximately 300 Gm., they were placed in metabolism cages and were adapted to the force-feeding of a medium carbohydrate diet

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† Thanks are due to Dr. K. K. Chen, The Lilly Research Laboratories, Indianapolis, Ind., who supplied the HPC for this study.

(Table I) by stomach tube each morning and late afternoon. The technique and diets were modified from those described by Reinecke, Ball, and Samuels (5). During the period of adaptation to force-feeding, the amount of diet was increased gradually to prevent the development of food shock. The animals were brought to a full feeding of 26 cc. of diet per day on the sixth day. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with toluene. Urine glucose was determined by the ferricyanide electrode method of Shaffer and Williams (6).

Each drug was ground into a fine suspension in peanut oil at a concentration of 100 mg./cc. It was administered by subcutaneous injection immediately after the morning and late afternoon feedings.

## EXPERIMENTS AND RESULTS

In experiment 1 (Fig. 1), 36 rats were observed during a control period of several weeks. Six severely diabetic rats were treated with aspirin in daily doses of 40, 80, and 160 mg. for seven days at each dose level. Twelve similar animals were given an identical course of HPC. Six moderately diabetic rats were given an identical course of aspirin and twelve similar animals were given an identical course of HPC. Aspirin suppressed the glycosuria of each rat to an extent that was proportional to the dose. When the injections of aspirin were stopped, there was an exacerbation of the glycosuria to levels much higher than observed in the initial control period. HPC was without significant effect upon the glycosuria of any of the rats. Six of the moder-

TABLE I.—MEDIUM CARBOHYDRATE DIET

| Constituent | Quantity, Gm. |
|---|---|
| Cellu flour (Chicago Dietetic Supply House) | 60 |
| Osborne & Mendel salt mixture | 40 |
| Dried yeast (Pabst) | 100 |
| Wheat germ oil | 10 |
| Cod liver oil | 10 |
| Vitamin K (2-methyl-1,4-naphthoquinone) | 0.1 |
| Mazola oil | 200 |
| Casein (Labco) | 160 |
| Starch | 200 |
| Dextrin | 190 |
| Sucrose | 200 |
| Water, to make total of: | 2,000 cc. |

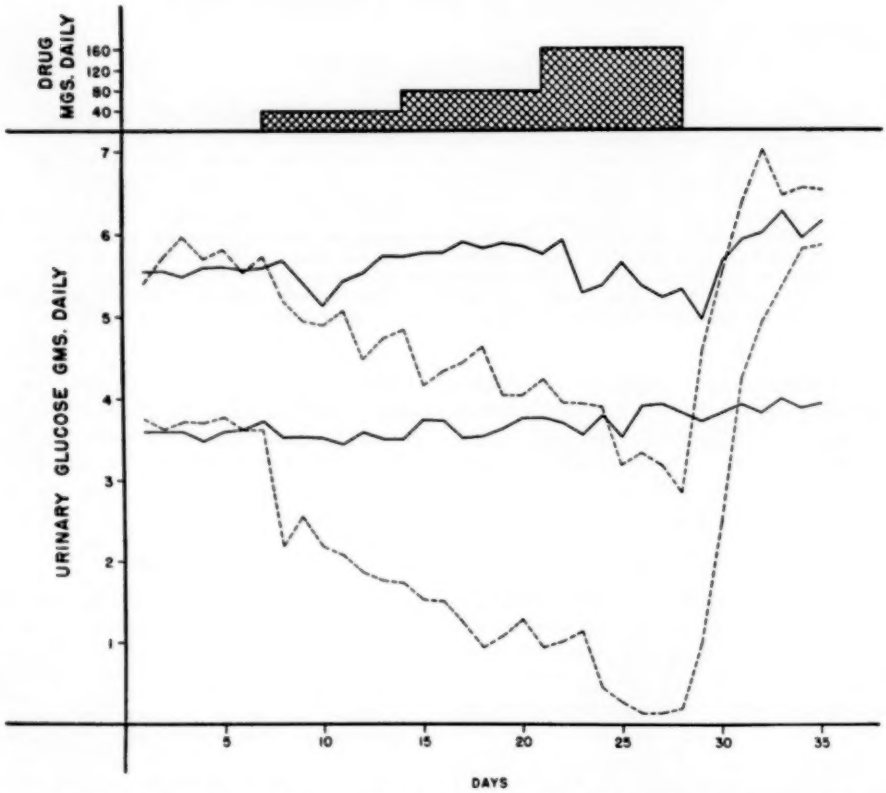


Fig. 1.—The effect of aspirin and HPC upon the level of glycosuria in the partially depancreatized, force-fed rat. Averages for 5 or 6 rats per group. ———HPC. ---Aspirin.

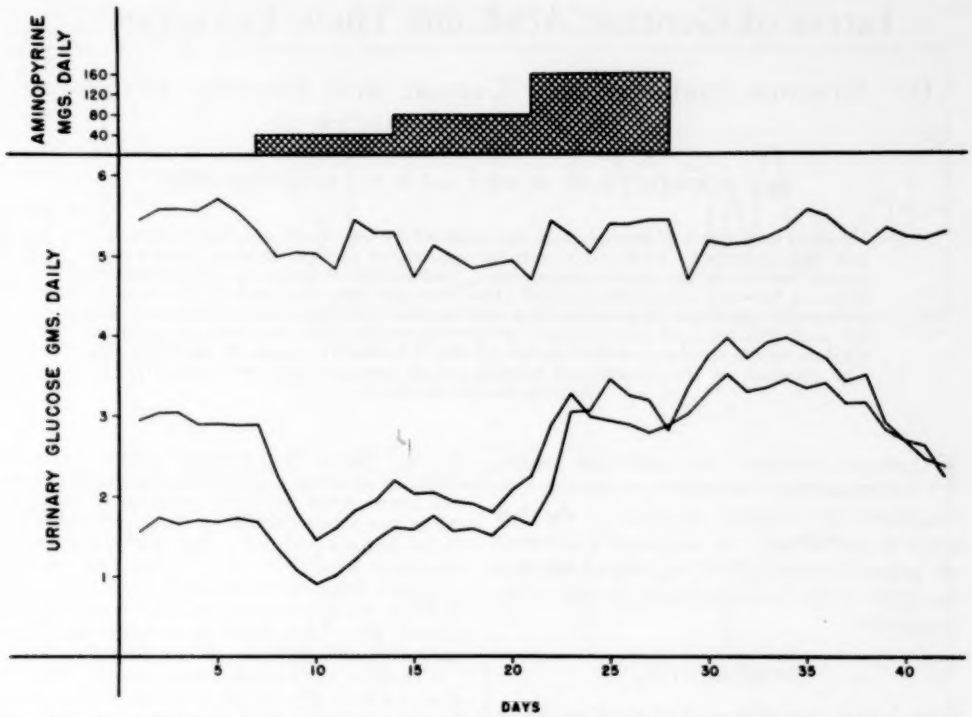


Fig. 2.—The effect of aminopyrine upon the level of glycosuria in the partially depancreatized, force-fed rat. Averages for 6 rats per group.

ately diabetic rats and 7 of the severely diabetic rats died during the administration of 160 mg. of HPC per rat per day. The values for urinary glucose are shown for the survivors (Fig. 1).

In experiment 2 (Fig. 2), the conditions of experiment 1 were repeated for 6 severely diabetic rats, 6 moderately diabetic rats, and 6 mildly diabetic rats. After a control period of several weeks, all of the rats were treated with aminopyrine at doses of 40, 80, and 160 mg. per rat per day for seven days at each dose. There was little effect of the aminopyrine upon the glycosuria of the severely diabetic rats but the remaining animals showed a temporary suppression of urinary glucose with a rise to the control level during continued treatment and with a temporary exacerbation of the glycosuria when the injections were stopped.

## DISCUSSION

These data support the results of a preliminary study (7) showing the effect of aspirin upon the glycosuria of the diabetic rat. Aminopyrine had a relatively small temporary effect upon the level of glycosuria and HPC was without any significant effect. The failure of these two drugs to reproduce the effect of aspirin upon the diabetic rat suggests that this property of aspirin is not based upon its antipyretic and analgesic properties since HPC and aminopyrine also possess them.

On the basis of these results it can be argued that these drugs do not cause a state of hypercorticalism in the diabetic rat since each fails to cause exacerbation of the diabetic state which is a usual consequence of experimental hypercorticalism. The fact that large doses of these drugs elicit a response in the anterior pituitary-adrenal cortex axis (1) is not in doubt but it has not been shown that the response is other than a nonspecific activation to the adrenal glands to meet an increased need for its hormones (8). Rall and Wells (9) have shown that HPC produces a significant reduction in pyrogen-induced fever in dogs under conditions in which corticotropin and cortisone were ineffective. These authors concluded that HPC does not exert an antipyretic action by virtue of its ability to cause a discharge of adrenal cortical hormones.

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# Esters of Gentisic Acid and Their Toxicities*,**

## II. Structure Studies of New Gentisic Acid Esters by Analyses of Their Infrared Spectra

By J. F. NASH,† F. W. BOPE,‡ and B. V. CHRISTENSEN§

**Thirteen new esters of gentisic acid are analyzed by use of infrared spectra to elucidate their structure. Only eight infrared spectra are shown, three of known compounds and five of the new synthetic esters. Analyses of the spectra indicate that the reaction between dialkylaminoalkyl chlorides and gentisic acid yields dialkylaminoalkyl gentisate hydrochlorides, and the reaction between gentisic acid and the acid chlorides of phenylacetic, anisic, *p*-nitrobenzoic, and succinic acids in alkaline media results in esterification of the 5-hydroxyl group of gentisic acid. The formula of the compound formed when gentisic acid and acetyl salicyl chloride react is in doubt.**

ELEMENTAL analyses and molecular weight determinations, reported previously (1), established the empirical formulas of the new esters of gentisic acid. In this paper, analyses of the infrared spectra of the compounds elucidate the nature of the functional groups present in the compounds.

### EXPERIMENTAL

The spectra were produced by a Baird Associates, Inc. Model B automatic recording infrared spectrophotometer. A standard liquid cell 0.10 mm. thick containing sodium chloride windows was used. The solvents used were chloroform, ether, and Nujol gel.

The infrared spectra of the synthesized esters were analyzed by comparing them with the infrared spectra of known compounds[1] and using the tables compiled by the Chemistry Department of The Ohio State University. The volume by Randall, *et al.* (2), was also consulted.

The spectra of thirteen new esters of gentisic acid and five known compounds were studied, but only eight typical spectra are reproduced in the present paper.

The two main features that are outstanding in each spectrum of the known compounds are the low transmissions in the ranges 3.0 to 4.0 $\mu$ and 5.6 to 6.0 $\mu$. In Fig. 1, the strong absorption in these regions indicates a phenolic group or groups and one carbonyl function. However, in Figs. 2 and 3 where the 5-hydroxyl group of gentisic acid has been modified, the nature of the absorption in the ranges 3.0 to 4.0 $\mu$ and 5.6 to 6.0 $\mu$ is markedly changed. In these figures the absorption is decreased in the range 3.0 to 4.0 $\mu$. The decreased absorption was also noted when an ether function, or methoxyl group, was present instead of the 5-hydroxyl group. In the range 5.6 to 6.0 $\mu$, Figs. 2 and 3 show two prominent absorption bands. The first "hump" represents the carbonyl function of the ester group in the number five position of gentisic acid, and the second "hump" represents the carbonyl function of the carboxylic acid group of gentisic acid.

Since these two regions, 3.0 to 4.0 $\mu$ and 5.6 to 6.0 $\mu$, are the ones of value for the proof of structure of the known compounds by an analysis of their spectra, the new esters of gentisic acid were evaluated in regard to these regions.

Figures 4 and 5 show much the same absorption patterns as Figs. 2 and 3. The absorption in the range 3.0 to 4.0 $\mu$ is low and the two carbonyl functions, which are in ester groups, are indicated by the two absorption peaks between 5.6 to 6.0 $\mu$. The absorption peak at 4.0 to 4.25 $\mu$ in each spectrum is believed to represent the hydrogen chloride of the compound.

Figure 6 shows only one carbonyl function, which represents the ester group. The strong absorption peaks at 3.5, 6.8, and 8.2 $\mu$ are from the Nujol in which the compound was incorporated for determining its spectrum. The background from the Nujol covers not only the blunt absorption peak due to the hydrogen chloride in the compound, but also the phenolic peak in the region 3.0 to 4.0 $\mu$.

The infrared spectra of 2-diethylaminoethyl gentisate hydrochloride, 2-diethylaminoethyl-5-methoxy salicylate hydrochloride, 3-diethylaminopropyl-5-acetyl-gentisate hydrochloride, 3-diethylaminopropyl-5-methoxy salicylate hydrochloride, and 3-diethylaminopropyl-5-benzoyl-gentisate hydrochloride were determined and analyzed in the same manner to elucidate the structures of the compounds.

The spectra of 5-phenylacetyl-gentisic acid and 5-anisyl-gentisic acid, Figs. 7 and 8, are very similar to the spectra of 5-acetyl-gentisic acid and 5-benzoyl-gentisic acid, Figs. 2 and 3. There is relatively weak absorption in the region 3.0 to 4.0 $\mu$, and two absorption peaks appear in the region 5.6 to 6.0 $\mu$. The carbonyl functions indicated by these two absorption peaks in the region 5.6 to 6.0 $\mu$ are part of the ester and carboxylic acid groups.

* Received August 22, 1952, from the College of Pharmacy, The Ohio State University, Columbus.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.
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§ Dean of the College of Pharmacy, The Ohio State University.
[1] Gentisic acid, 5-acetyl-gentisic acid, 5-methoxy-salicylic acid, diacetyl-gentisic acid, and 5-benzoyl-gentisic acid.

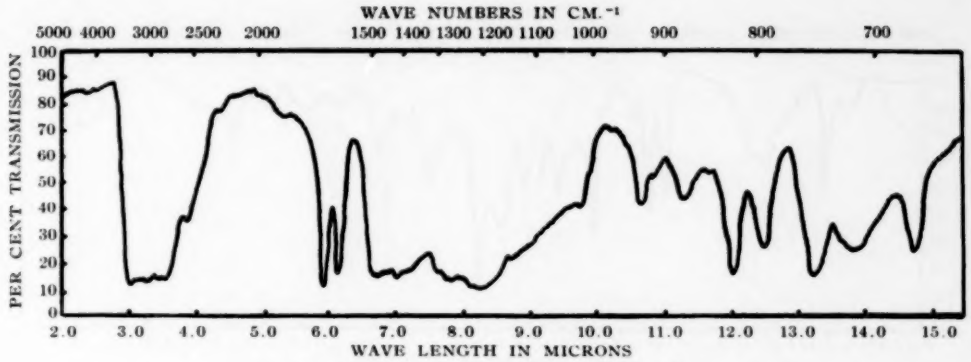


Fig. 1.—Infrared spectrum of gentisic acid.

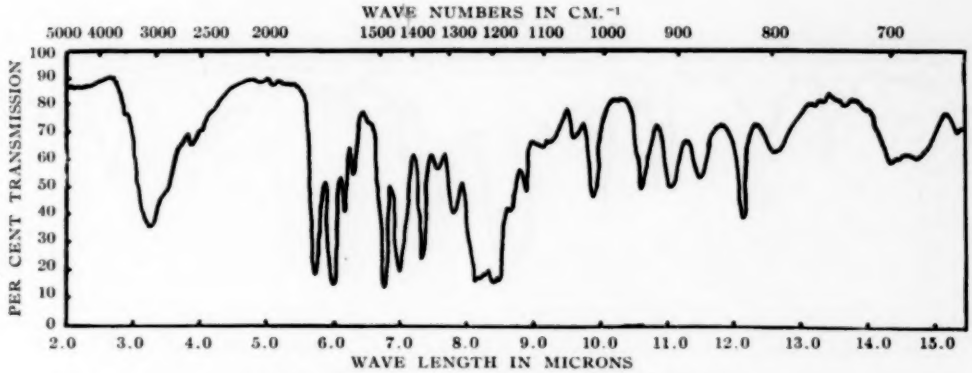


Fig. 2.—Infrared spectrum of 5-acetyl gentisic acid.

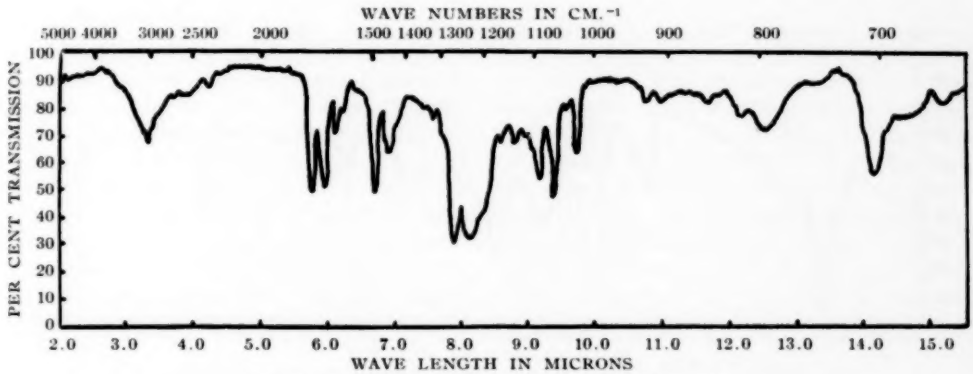


Fig. 3.—Infrared spectrum of 5-benzoyl gentisic acid.

The infrared spectra of 5-*p*-nitrobenzoyl-gentisic acid and 5-5′-succinyl-digentisic acid were determined and analyzed. The spectrum of each compound is much the same as those shown in Figs. 7 and 8, and in addition, the nitro group is prominent. However, the spectrum of 5-acetylsalicyl-gentisic acid indicates only one carbonyl group. The formula of this latter compound is thus doubtful.

## SUMMARY AND CONCLUSIONS

1. The infrared spectra of thirteen new esters of gentisic acid were determined and analyzed to elucidate the structure of each of the compounds.

2. Esters of gentisic acid and amino alcohols: the reaction between dialkylamino-

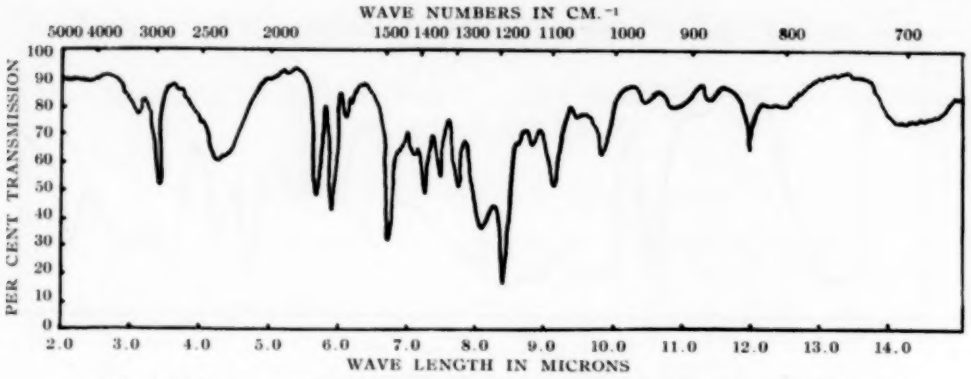


Fig. 4.—Infrared spectrum of 2-diethylaminoethyl-5-acetyl gentisate hydrochloride.

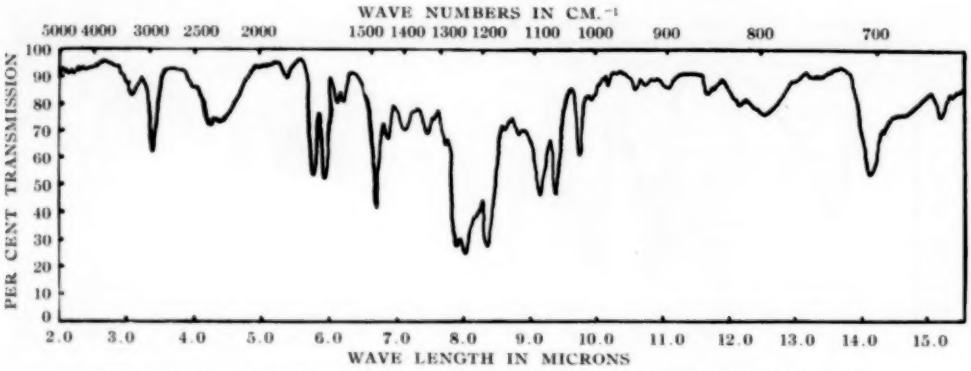


Fig. 5.—Infrared spectrum of 2-diethylaminoethyl-5-benzoyl gentisate hydrochloride.

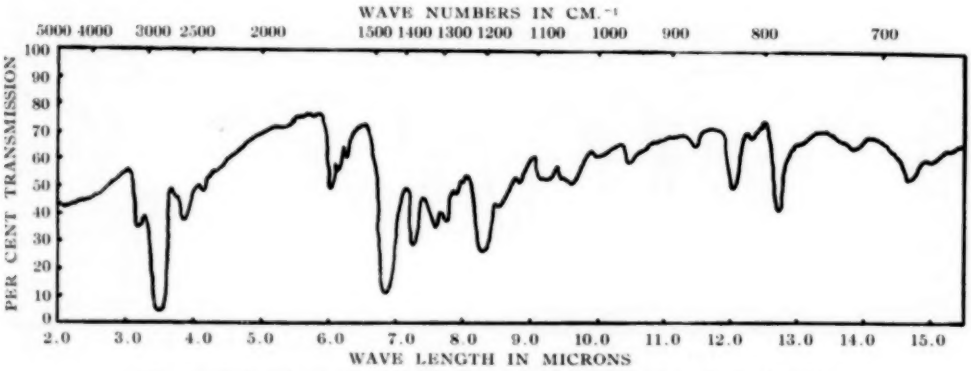


Fig. 6.—Infrared spectrum of 3-diethylaminopropyl gentisate hydrochloride.

alkyl chlorides and gentisic acid yields dialkylaminoalkyl gentisate hydrochlorides. 2-Diethylaminoethyl gentisate hydrochloride, 2-diethylaminoethyl-5-methoxy-salicylate hydrochloride, and the 3-diethylaminopropyl counterparts show only one carbonyl function in each spectrum. This indicates esterification through the carboxylic acid group of gentisic acid. 2-Diethylaminoethyl-5-acetyl-gentisate hydrochloride, 2-diethylaminoethyl-5-benzoyl-gentisate hydrochloride, and the 3-diethylaminopropyl counterparts show two carbonyl functions in each spectrum. This indicates esterification of the carboxylic acid group and also the 5-hydroxyl group

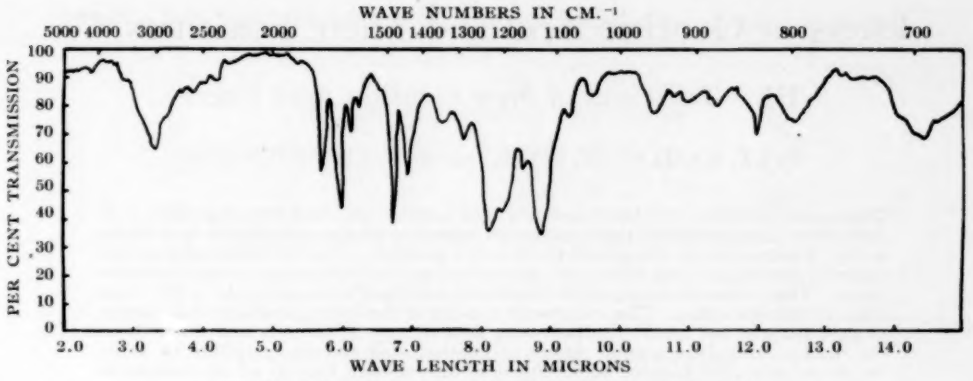


Fig. 7.—Infrared spectrum of 5-phenylacetyl gentisic acid.

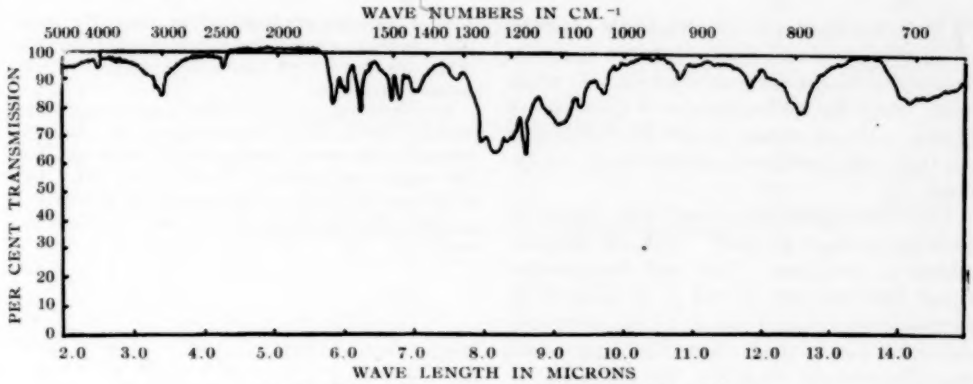


Fig. 8.—Infrared spectrum of 5-anisyl gentisic acid.

of gentisic acid. The hydrogen chloride group is prominent in each spectrum except those in which Nujol was the medium for spectrum determination. In these cases, the absorption of Nujol was too great to permit the detection of the hydrogen chloride group. The 5-hydroxyl group of gentisic acid esters appears to have a more pronounced absorption than the 2-hydroxyl group. This is indicated by the fact that modification of the 5-position greatly reduces absorption in the "phenolic range" of the infrared spectra of these compounds.

3. Esters of gentisic acid and other carboxylic acids: the reaction between gentisic acid and the acid chlorides of phenylacetic, anisic, *p*-nitrobenzoic, and succinic acids in alkaline media results in esterification of the 5-hydroxyl group of gentisic acid. With the exception of 5-acetylsalicyl-gentisic acid, the esters show two carbonyl functions in each spectrum. This indicates that the esterification of gentisic acid took place between the acid chloride and the 5-hydroxyl group of gentisic acid. The spectrum of 5-acetylsalicyl-gentisic acid shows only one carbonyl function; therefore, doubt exists as to whether the proposed formula indicates the true nature of the compound.

## REFERENCES

(1) Nash, J. F., Bope, F. W., and Christensen, B. V., THIS JOURNAL, **42**, 207(1953).

(2) Randall, H. M., Fowler, L. D., Fuson, N., and Dangl, J. R., "Infrared Determination of Organic Molecules," D. Van Nostrand Co., New York, 1941, pp. 10–20.

# Esters of Gentisic Acid and Their Toxicities*,**

## III. Toxicities of New Gentisic Acid Esters

By J. F. NASH,† F. W. BOPE,‡ and B. V. CHRISTENSEN§

**The acute toxicities of thirteen new esters of gentisic acid and that of gentisic acid have been determined by the intravenous injection of the compounds into white mice. The results are integrated by Behren's method. The 3-diethylaminopropyl esters of gentisic acid are more toxic generally than the 2-diethylaminoethyl counterparts. The 3-diethylaminopropyl-5-methoxy salicylate hydrochloride is the most toxic of the new esters. The increase in toxicity of the hydrochlorides of the esters of gentisic acid and amino alcohols over gentisic acid apparently is due chiefly to the dialkylaminoalkyl group. Esters of gentisic acid and other carboxylic acids are more toxic than gentisic acid because of the inherent toxicity of the carboxylic acids. These compounds are less toxic than the esters of gentisic acid and amino alcohols because they are less soluble in aqueous media.**

THE acute toxicities of thirteen new esters of gentisic acid and that of gentisic acid were determined by intravenous injection into white mice. Since the hydrochlorides of the esters of gentisic acid and amino alcohols are water soluble, these compounds were administered as solutions.

The esters of gentisic acid and other carboxylic acids are insoluble in water. Although they are soluble in propylene glycol and polyethylene glycol, both solvents proved to be toxic when administered in doses as low as 0.1 ml. per mouse, and could not be used. Therefore suspensions were prepared by dissolving the compound in alcohol, adding an amount of Tween 80 corresponding to 3 per cent of the alcohol solution, and then adding this mixture to water containing not more than 5 per cent acacia. The total alcohol content was not more than 5 per cent, and the suspension was administered intravenously.

### PROCEDURE

Swiss mice, all from the same source of supply and weighing from 10 to 28 Gm., were used. They were in good health and had been kept under observation for at least three days prior to use. The mice were housed in standard cages in a room with nearly constant temperature and humidity. Their diet consisted of an unlimited ration of water and Purina Dog Checkers. They were starved twenty-four hours prior to use in order to standardize their metabolism.

All doses were administered on a mg./Kg. basis. The solutions were injected slowly into the tail veins of the mice by a 1-ml. tuberculin syringe with a 25 gauge, 1-in. needle.

After determining the toxicity ranges of each compound, the essential requirements for Behren's method of statistical evaluation (1) were satisfied. The results are shown in Tables I and III. This integrated data is illustrated graphically in Figs. 1–4, and the $LD_{50}$ of each ester is reported in Tables II and IV.

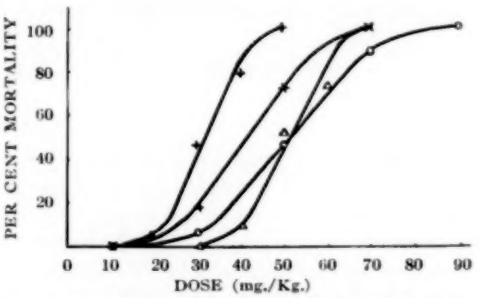


Fig. 1.—Dosage-mortality curves of 2-diethylaminoethyl esters of gentisic acid.

○: 2-Diethylaminoethyl gentisate hydrochloride. Δ: 2-Diethylaminoethyl-5-acetyl gentisate hydrochloride. +: 2-Diethylaminoethyl-5-methoxy salicylate hydrochloride. X: 2-Diethylaminoethyl-5-benzoyl gentisate hydrochloride.

The least toxic of the compounds (Table II) was 3-diethylaminopropyl gentisate hydrochloride with an $LD_{50}$ of 58.5 mg./Kg., and the most toxic was 3-diethylaminopropyl-5-methoxy salicylate hydrochloride with an $LD_{50}$ of 22.0 mg./Kg.

Generally, the 3-diethylaminopropyl ester hydrochlorides were more toxic than the 2-diethylaminoethyl compounds, although the first members of the series are reversed in this respect. The most toxic esters were those in which the 5-phenolic hydroxyl groups had been methylated.

* Received August 22, 1952, from the College of Pharmacy, The Ohio State University, Columbus.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.
** Abstract of a dissertation submitted to the Graduate School of The Ohio State University by J. F. Nash in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Present address: Eli Lilly and Co., Research Laboratories, Indianapolis, Ind.
‡ Associate Professor of Pharmacy, The Ohio State University.
§ Dean of the College of Pharmacy, The Ohio State University.

TABLE I.—TOXICITY DETERMINATIONS OF ESTERS OF GENTISIC ACID AND AMINO ALCOHOLS

| No. of Animals | Dose, Mg./Kg. | Experimental Data | | | Integrated Data | | |
|---|---|---|---|---|---|---|---|
| | | Alive | Dead | Mortality, % | Alive | Dead | Mortality, % |
| **2-Diethylaminoethyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 10 | 12 | 0 | 0 | 31 | 0 | 0 |
| 12 | 30 | 11 | 1 | 8 | 19 | 1 | 5 |
| 12 | 50 | 6 | 6 | 50 | 8 | 7 | 47 |
| 12 | 70 | 2 | 10 | 83 | 2 | 17 | 89 |
| 12 | 90 | 0 | 12 | 100 | 0 | 20 | 100 |
| **2-Diethylaminoethyl-5-Acetyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 30 | 12 | 0 | 0 | 31 | 0 | 0 |
| 12 | 40 | 10 | 2 | 17 | 19 | 2 | 9 |
| 12 | 50 | 4 | 8 | 67 | 9 | 10 | 53 |
| 12 | 60 | 5 | 7 | 58 | 5 | 17 | 74 |
| 12 | 70 | 0 | 12 | 100 | 0 | 29 | 100 |
| **2-Diethylaminoethyl-5-Methoxy Salicylate Hydrochloride** | | | | | | | |
| 12 | 10 | 12 | 0 | 0 | 32 | 0 | 0 |
| 12 | 20 | 11 | 1 | 8 | 20 | 1 | 5 |
| 12 | 30 | 5 | 7 | 58 | 9 | 8 | 47 |
| 12 | 40 | 4 | 8 | 67 | 4 | 16 | 80 |
| 12 | 50 | 0 | 12 | 100 | 0 | 28 | 100 |
| **2-Diethylaminoethyl-5-Benzoyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 10 | 12 | 0 | 0 | 25 | 0 | 0 |
| 12 | 30 | 9 | 3 | 25 | 13 | 3 | 19 |
| 12 | 50 | 4 | 8 | 67 | 4 | 11 | 73 |
| 12 | 70 | 0 | 12 | 100 | 0 | 23 | 100 |
| **3-Diethylaminopropyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 30 | 12 | 0 | 0 | 23 | 0 | 0 |
| 12 | 50 | 8 | 4 | 33 | 11 | 4 | 27 |
| 12 | 70 | 3 | 9 | 75 | 3 | 13 | 81 |
| 12 | 90 | 0 | 12 | 100 | 0 | 25 | 100 |
| **3-Diethylaminopropyl-5-Acetyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 30 | 12 | 0 | 0 | 23 | 0 | 0 |
| 12 | 40 | 5 | 7 | 58 | 11 | 7 | 39 |
| 12 | 50 | 4 | 8 | 67 | 6 | 15 | 71 |
| 12 | 60 | 2 | 10 | 83 | 2 | 25 | 93 |
| 12 | 70 | 0 | 12 | 100 | 0 | 37 | 100 |
| **3-Diethylaminopropyl-5-Methoxy Salicylate Hydrochloride** | | | | | | | |
| 12 | 10 | 12 | 0 | 0 | 22 | 0 | 0 |
| 12 | 20 | 7 | 5 | 42 | 10 | 5 | 33 |
| 12 | 30 | 2 | 10 | 83 | 3 | 15 | 83 |
| 12 | 40 | 1 | 11 | 92 | 1 | 26 | 96 |
| 12 | 50 | 0 | 12 | 100 | 0 | 38 | 100 |
| **3-Diethylaminopropyl-5-Benzoyl Gentisate Hydrochloride** | | | | | | | |
| 12 | 20 | 13 | 0 | 0 | 31 | 0 | 0 |
| 12 | 25 | 11 | 1 | 8 | 19 | 1 | 5 |
| 12 | 30 | 6 | 6 | 50 | 8 | 7 | 47 |
| 12 | 35 | 2 | 10 | 83 | 2 | 17 | 89 |
| 12 | 40 | 0 | 12 | 100 | 0 | 29 | 100 |

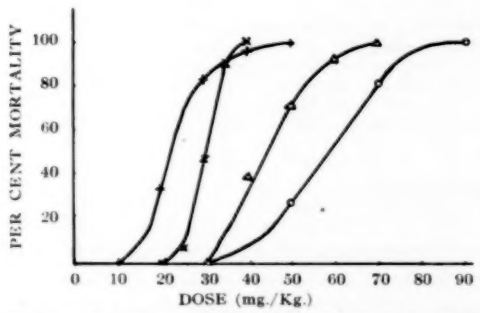


Fig. 2.—Dosage-mortality curves of 3-diethylaminopropyl esters of gentisic acid.

O: 3-Diethylaminopropyl gentisate hydrochloride. Δ: 3-Diethylaminopropyl-5-acetyl gentisate hydrochloride. +: 3-Diethylaminopropyl-5-methoxy salicylate hydrochloride. X: 3-Diethylaminopropyl-5-benzoyl gentisate hydrochloride.

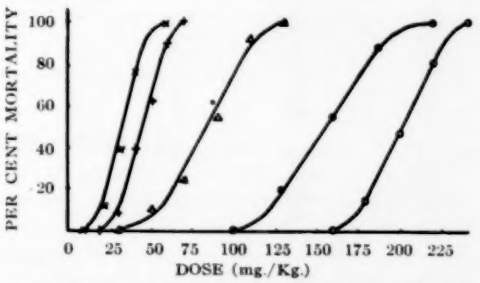


Fig. 3.—Dosage-mortality curves of esters of gentisic acid and other carboxylic acids.

O: 5-Phenylacetyl gentisic acid. Δ: 5-*p*-Nitrobenzoyl gentisic acid. +: 5-Anisyl gentisic acid. X: 5-Acetylsalicyl gentisic acid. ●: 5,5′-Succinyl digentisic acid.

It is assumed that the toxicity of these compounds is due principally to the presence of the dialkyl-

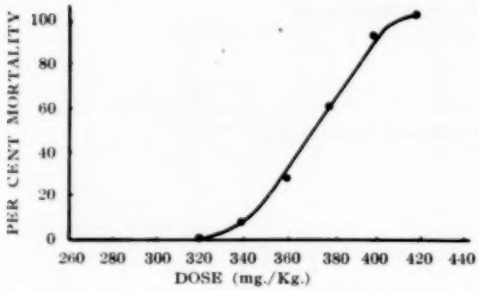


Fig. 4.—Dosage-mortality curve of gentisic acid.

aminoalkyl groups, since the parent compounds are relatively nontoxic.

The mice that died went into convulsions and died within one minute after the compound was injected. The remaining mice were observed for twenty-four hours following administration of the drugs, but no additional deaths occurred.

TABLE II.—$LD_{50}$ OF HYDROCHLORIDES OF ESTERS OF GENTISIC ACID AND AMINO ALCOHOLS

| Name of Compound | $LD_{50}$, Mg./Kg. |
|---|---|
| 2-Diethylaminoethyl gentisate hydrochloride | 51.2 |
| 2-Diethylaminoethyl-5-acetyl gentisate hydrochloride | 51.5 |
| 2-Diethylaminoethyl-5-methoxy salicylate hydrochloride | 32.2 |
| 2-Diethylaminoethyl-5-benzoyl gentisate hydrochloride | 41.7 |
| 3-Diethylaminopropyl gentisate hydrochloride | 58.5 |
| 3-Diethylaminopropyl-5-acetyl gentisate hydrochloride | 44.5 |
| 3-Diethylaminopropyl-5-methoxy salicylate hydrochloride | 22.0 |
| 3-Diethylaminopropyl-5-benzoyl gentisate hydrochloride | 30.3 |

TABLE III.—TOXICITY DETERMINATIONS OF ESTERS OF GENTISIC ACID AND OTHER CARBOXYLIC ACIDS

| No. of Animals | Experimental Data: Dose, Mg./Kg. | Alive | Dead | Mortality, % | Integrated Data: Alive | Dead | Mortality, % |
|---|---|---|---|---|---|---|---|
| | | | **5-Phenylacetyl Gentisic Acid** | | | | |
| 12 | 160 | 12 | 0 | 0 | 31 | 0 | 0 |
| 12 | 180 | 9 | 3 | 25 | 19 | 3 | 14 |
| 12 | 200 | 6 | 6 | 50 | 10 | 9 | 47 |
| 12 | 220 | 4 | 8 | 67 | 4 | 17 | 81 |
| 12 | 240 | 0 | 12 | 100 | 0 | 29 | 100 |
| | | | **5-*p*-Nitrobenzoyl Gentisic Acid** | | | | |
| 12 | 30 | 12 | 0 | 0 | 39 | 0 | 0 |
| 12 | 50 | 9 | 3 | 25 | 27 | 3 | 10 |
| 12 | 70 | 9 | 3 | 25 | 18 | 6 | 25 |
| 12 | 90 | 7 | 5 | 42 | 9 | 11 | 55 |
| 12 | 110 | 2 | 10 | 83 | 2 | 21 | 92 |
| 12 | 130 | 0 | 12 | 100 | 0 | 33 | 100 |
| | | | **5-Anisyl Gentisic Acid** | | | | |
| 12 | 20 | 12 | 0 | 0 | 36 | 0 | 0 |
| 12 | 30 | 10 | 2 | 17 | 24 | 2 | 8 |
| 12 | 40 | 5 | 7 | 58 | 14 | 9 | 39 |
| 12 | 50 | 6 | 6 | 50 | 9 | 15 | 62 |
| 12 | 60 | 3 | 9 | 75 | 3 | 24 | 89 |
| 12 | 70 | 0 | 12 | 100 | 0 | 36 | 100 |
| | | | **5-Acetylsalicyl Gentisic Acid** | | | | |
| 12 | 10 | 12 | 0 | 0 | 33 | 0 | 0 |
| 12 | 20 | 9 | 3 | 25 | 21 | 3 | 12 |
| 12 | 30 | 7 | 5 | 42 | 12 | 8 | 40 |
| 12 | 40 | 4 | 8 | 67 | 5 | 16 | 76 |
| 12 | 50 | 1 | 11 | 92 | 1 | 27 | 96 |
| 12 | 60 | 0 | 12 | 100 | 0 | 39 | 100 |
| | | | **5,5′-Succinyl Digentisic Acid** | | | | |
| 12 | 100 | 12 | 0 | 0 | 28 | 0 | 0 |
| 12 | 130 | 8 | 4 | 33 | 16 | 4 | 20 |
| 12 | 160 | 6 | 6 | 50 | 8 | 10 | 55 |
| 12 | 190 | 2 | 10 | 93 | 2 | 20 | 91 |
| 12 | 220 | 0 | 12 | 100 | 0 | 32 | 100 |
| | | | **Gentisic Acid** | | | | |
| 12 | 320 | 12 | 0 | 0 | 38 | 0 | 0 |
| 12 | 340 | 10 | 2 | 17 | 26 | 2 | 7 |
| 12 | 360 | 8 | 4 | 33 | 16 | 6 | 27 |
| 12 | 380 | 6 | 6 | 50 | 8 | 12 | 60 |
| 12 | 400 | 2 | 10 | 83 | 2 | 22 | 92 |
| 12 | 420 | 0 | 12 | 100 | 0 | 34 | 100 |

The esters listed in Table IV vary in toxicity from 32.4 mg./Kg. for 5-acetylsalicyl gentisic acid to 201.2 mg./Kg. for 5-phenylacetyl gentisic acid, and all caused convulsions before death. However, these compounds were slower-acting than those listed in Table II, and generally required several minutes after injection to kill the animal.

Since the $LD_{50}$ for gentisic acid was found to be 374.0 mg./Kg. when administered intravenously, the toxicities of the synthesized esters are thus considerably greater than gentisic acid.

TABLE IV.—$LD_{50}$ OF ESTERS OF GENTISIC ACID AND OTHER CARBOXYLIC ACIDS

| Name of Compound | $LD_{50}$, Mg./Kg. |
|---|---|
| 5-Phenylacetyl gentisic acid | 201.2 |
| 5-*p*-Nitrobenzoyl gentisic acid | 83.5 |
| 5-Anisyl gentisic acid | 45.0 |
| 5-Acetylsalicyl gentisic acid | 32.4 |
| 5,5′-Succinyl digentisic acid | 155.0 |

Generally, the toxicities of the esters of gentisic acid and other carboxylic acids are lower than those of the esters of gentisic acid and amino alcohols, possibly due to the fact that the former were administered as suspensions whereas the latter were given as aqueous solutions.

Since the new esters show higher toxicities, they must possess much more activity than gentisic acid to be of greater medicinal value.

## SUMMARY AND CONCLUSIONS

1. The $LD_{50}$ of each new ester of gentisic acid was determined by intravenous injection of the compounds into white mice.

2. Of the hydrochlorides of the esters of gentisic acid and amino alcohols, the least toxic was 3-diethylaminopropyl gentisate hydrochloride with an $LD_{50}$ of 58.5 mg./Kg. in mice, whereas the most toxic was 3-diethylaminopropyl-5-methoxy salicylate hydrochloride with an $LD_{50}$ of 22.0 mg./Kg. The 3-diethylaminopropyl ester hydrochlorides were more toxic than the 2-diethylaminoethyl ester hydrochlorides of the same compounds.

3. Of the esters of gentisic acid and other carboxylic acids, 5-phenylacetyl gentisic acid, with an $LD_{50}$ of 201.2 mg./Kg., was the least toxic and 5-acetylsalicyl gentisic acid, with an $LD_{50}$ of 32.4 mg./Kg., was the most toxic. Substitution on the aromatic ring of the carboxylic acids esterified with gentisic acid seems to increase the toxicity of this group of esters greatly.

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(1) Behren, B., *Arch. Exptl. Path. Pharmakol.*, **140**, 237 (1929).
(2) Ginoulhiac, E., Semenza, F., and Mainardi, L., *Boll. soc. ital. biol. sper.*, **26**, 241(1950).

# The Assay for the Histamine-like Activity of Some Antibiotics*

By L. W. ROWE and R. A. BROWN†

**It is shown that dogs, although appreciably less sensitive to histamine than cats, are suitable for the assay of possible histamine-like activity as a trace contaminant of some antibiotics. Several techniques have been used to try to distinguish between actual histamine and histamine-like activity of highly purified antibiotics and some cruder experimental preparations. Standard errors are given for the assay of histamine-like activity in a variety of antibiotic preparations.**

THE PRESENCE of histamine or histamine-like substances as undesirable trace contaminants in the earlier antibiotic preparations was recognized as a distinct possibility because these antibiotics were prepared by fermentation processes. With the chemical purification of antibiotics of fermentation origin such as penicillin, streptomycin, dihydrostreptomycin, viomycin, and Chloromycetin[1] to the point of producing crystalline material and the successful synthetic production of Chloromycetin as well, the possibility of histamine or histamine-like substances being present even in minute quantities became very remote indeed.

* Received August 22, 1952, from the Research Laboratories of Parke, Davis and Co., Detroit 32, Mich.
† With the technical assistance of H. W. Pfeifle.

[1] Parke, Davis and Co. trade-mark name for chloramphenicol.

However, it is still considered desirable to apply safety tests to some of the antibiotics and their combinations for traces of histamine or histamine-like substances. The present report is a brief summary of experimental work done on this subject, using at least 100 dogs and about 50 cats in the experiments.

## EXPERIMENTAL

Since histamine causes definite but not maximal contraction of isolated strips of guinea-pig uterus in dilutions as high as one part in one hundred million, this pharmacological method was formerly used (1, 2) for determining the amount of histamine present as an impurity in endocrine gland extracts. Because the action of histamine on the guinea-pig uterus is not specific, and to differentiate between the action of the oxytocic hormone of the posterior pituitary and that of histamine, the glandular extract was treated with strong alkali for a short time to destroy the oxytocin but not the histamine.

Depressor methods (3, 4) using anesthetized dogs and cats had also been employed to determine histamine since it has a powerful and rather transient vasodilator action when very small doses are given intravenously. With the advent of streptomycin, the Food and Drug Administration of the Federal Security Agency, on April 4, 1947, in the Federal Register (5, 6), issued the details of an official safety method for histamine in preparation of this antibiotic. Adult cats anesthetized with sodium phenobarbital given intraperitoneally were recommended for this depressor test, but the following statement was appended: "Dogs may be substituted for cats in this test provided the ratio of the doses of streptomycin and histamine employed is the same." The product is considered satisfactory if the fall in blood pressure obtained with 3 mg. of streptomycin per Kg. of body weight (a short time later this was revised upward to 30 mg./Kg.) is no greater than the fall obtained with 0.10 μg. of histamine base per Kg. of body weight.

Because of the preference of the F. D. A. for the cat depressor method, its use has been continued for histamine assays. Its only advantage, however, is the greater sensitivity of cats to histamine while the disadvantages of unsatisfactory and limited animal supply and greater difficulties in maintaining and handling these animals in the laboratory more than offset the doubtful advantage. Our experience with dogs indicates that a medium sized male (7–10 Kg.), when anesthetized with sodium phenobarbital (140 mg./Kg. given intraperitoneally; other anesthetics, especially chloretone, are not so satisfactory) will usually show a depressor response of 20 mm. or more from an intravenous dose of 0.10 cc./Kg. of a 1:200,000 solution of histamine base or 0.50 μg./Kg. The average cat will show a similar response from a 1:1,000,000 solution of histamine base but this five-times greater sensitivity is of no real advantage, since all reactions must be compared directly with the standard on the same animal during a particular test.

Table I summarizes some of the data obtained on dogs and cats showing their comparative dose-response and its slope.

TABLE I.—DEPRESSOR RESPONSE OF ANESTHESIZED DOGS AND CATS TO HISTAMINE BASE

| Animal Wt., Kg. | Anesthetic | Histamine Dose, μg./Kg. (I.V.) | Response Av., Mm. | Range μg./Kg./Mm. | Slope |
|---|---|---|---|---|---|
| Dog 7.4 | Phenobarbital | 0.50 | 17 | 0.25 = 10 ...<br>0.50 = 18 ...<br>0.75 = 24 ... | 29.07 |
| Dog 6.0 | Phenobarbital | 0.50 | 13 | 0.25 = 9 ...<br>0.50 = 18 ...<br>0.75 = 22 ... | 27.55 |
| Dog 10.4 | Phenobarbital | 0.50 | 27 | 0.25 = 16 ...<br>0.50 = 25 ...<br>0.75 = 34 ... | 36.92 |
| Dog 6.4 | Phenobarbital | 0.50 | 32.4 | 0.25 = 18 ...<br>0.50 = 33 ...<br>0.75 = 39 ... | 44.68 |
| Dog 7.8 | Phenobarbital | 0.50 | 33 | 0.25 = 28 ...<br>0.50 = 36 ...<br>0.75 = 42 ... | 29.09 |
| Dog 10.4 | Phenobarbital | 0.25 | 36.6 | | |
| Dog 5.2 | Phenobarbital | 0.50 | 29 | | |
| Dog 6.2 | Phenobarbital | 0.50 | 27 | 0.25 = 12 ...<br>0.50 = 27 ...<br>0.75 = 34 ... | 46.55 |
| Dog 9.8 | Phenobarbital | 0.25 | 26 | 0.25 = 22 ...<br>0.50 = 45 ...<br>0.75 = 54 ... | 68.13 |
| Dog 7.8 | Phenobarbital | 0.50 | 21 | 0.25 = 11 ...<br>0.50 = 18 ...<br>0.75 = 25 ... | 28.71 |

(*Continued on page 259*)

TABLE I (*Continued from page 258*)

| Animal Wt., Kg. | Anesthetic | Histamine Dose, μg./Kg. (I.V.) | Response Av., Mm. | Range μg./Kg./Mm. | Slope |
|---|---|---|---|---|---|
| Dog 10.6 | Phenobarbital | 0.50 | 24.6 | 0.25 = 21 ...<br>0.50 = 30 ...<br>0.75 = 37 ... | 33.18 |
| Dog 9.0 | Phenobarbital | 0.50 | 30.4 | 0.25 = 21<br>0.50 = 34 Average:<br>0.75 = 39 | 38.22 |
| Dog 10.4 | Phenobarbital | 0.50 | 24 | | |
| Dog 9.0 | Phenobarbital | 0.50 | 30.7 | | |
| Dog 8.8 | Phenobarbital | 0.4 Hist. Base<br>0.5 Hist. Base<br>0.6 Hist. Base | | ... 23<br>... 27<br>... 29 | 34.36 |
| Cat 3.1 | Phenobarbital | 0.08 Hist. Base<br>0.10 Hist. Base<br>0.12 Hist. Base | | ... 27<br>... 31<br>... 33 | 34.36 |
| Cat 3.8 | Phenobarbital | 0.10 | 18 | 0.05 = 7 ...<br>0.10 = 16 ...<br>0.15 = 22 ... | 31.26 |
| Cat 3.6 | Phenobarbital | 0.05 | 26 | 0.05 = 21 ...<br>0.10 = 36 ...<br>0.15 = 47 ... | 37.30 |
| Cat 4.0 | Phenobarbital | 0.05 | 27 | 0.05 = 17 ...<br>0.10 = 28 ...<br>0.15 = 34 ... | 35.71 |
| Cat 3.0 | Phenobarbital | 0.15 | 18 | 0.05 = 8 ...<br>0.10 = 18 ...<br>0.15 = 24 ... | 33.49 |
| Cat 2.9 | Phenobarbital | 0.15 | 22 | 0.05 = 11 ...<br>0.10 = 22 ...<br>0.15 = 24 ... | 28.24 |
| Cat 3.3 | Phenobarbital | 0.10 | 22 | 0.05 = 9 ...<br>0.10 = 22 ...<br>0.15 = 32 ... | 47.65 |
| Cat 2.9 | Phenobarbital | 0.10 | 24.8 | | |
| Cat 3.4 | Phenobarbital | 0.10 | 24.4 | 0.05 = 24 ...<br>0.10 = 32 ...<br>0.15 = 42 ... | 36.54 |
| Cat 3.3 | Phenobarbital | 0.15 | 21 | | |
| Cat 3.8 | Phenobarbital | 0.10 | 21.6 | | |
| Cat 3.4 | Phenobarbital | 0.10 | 22.4 | | |
| Cat 3.0 | Phenobarbital | 0.10 | 21 | | |
| | | | | Average: | 35.74 |

Dogs anesthetized with sodium pentobarbital are not satisfactory for the assay of histamine, due to difficulty in maintaining uniform anesthesia and a lower degree of sensitivity. While dogs anesthetized with sodium phenobarbital are less sensitive to histamine than are cats (about one-fifth), the slope of the dose-response curve of medium sized males compares very favorably with that obtained from cats under similar anesthesia and such dogs are, therefore, entirely satisfactory for histamine assay purposes.

Table II indicates some typical data collected from tests on five antibiotics.

Experimental data that cannot be properly tabulated have been obtained on histamine and some of the antibiotics combined with small amounts of the antihistamines, Benadryl[2] and Ambodryl.[2] It was found that these antihistamines do not neutralize the action of histamine itself or the histamine-like activity of the antibiotics when they are first given to an animal as combinations. Depressor reactions from subsequent similar doses of such combinations or of histamine alone are definitely reduced. The antihistamines are, therefore, of no assistance in differentiating between a true histamine action and histamine-like action.

Table III shows that histaminase does destroy depressor activity known to be due to histamine itself but does not appreciably affect the histamine-like depressor activity of some antibiotics. Most

[2] Parke, Davis and Co. trade-mark names.

TABLE II.—ASSAYS OF SOME ANTIBIOTICS FOR HISTAMINE-LIKE ACTIVITY

| Product | Method | Results: Units or μg. = μg. Hist. Base |
|---|---|---|
| Penicillin 086104A | Cat Depressor | 4,000 < 0.10 |
| Penicillin 086499C | Cat Depressor | 4,000 < 0.10 |
| Penicillin 086498F | Cat Depressor | 4,000 < 0.10 |
| Penicillin 236-D-127 | Dog Depressor | 9,627 = 0.10 ± 4.22% |
| Penicillin 236-D-127 | Cat Depressor | 12,624 = 0.10 ± 7.21% |
| Streptomycin 7RI544 | Cat Depressor | 4,280 = 0.10 |
| Streptomycin 04068A | Cat Depressor | 5,250 = 0.10 |
| Streptomycin 99122 | Cat Depressor | 6,250 = 0.10 |
| Streptomycin 120X621 | Cat Depressor | 1,200 = 0.10 |
| Streptomycin 120X621 | Dog Depressor | 800 = 0.10 |
| Streptomycin 120X55H | Dog Depressor | 6,670 = 0.10 |
| Streptomycin 120X58J | Dog Depressor | 2,860 = 0.10 |
| Streptomycin X2646 | Cat Depressor | 12,000 = 0.10 |
| Streptomycin X2648 | Cat Depressor | 12,000 = 0.10 |
| Streptomycin X2649 | Cat Depressor | 15,000 = 0.10 |
| Dihydrostreptomycin Sulf. A | Cat Depressor | 33,970 = 0.10 ± 1.99% |
| Dihydrostreptomycin Sulf. A | Dog Depressor | 10,836 = 0.10 ± 15.2% |
| Dihydrostreptomycin Sulf. A | Dog Depressor | 5,358 = 0.10 ± 6.85% |
| Dihydrostreptomycin Sulf. A | Cat Depressor | 7,890 = 0.10 ± 7.21% |
| Viomycin X4381 | Uterine Strip | 800 = 0.10 |
| Viomycin X4381 | Cat Depressor | 13,640 = 0.10 ± 16.7% |
| Viomycin X4381 | Dog Depressor | 2,778 = 0.10 ± 3.81% |
| Viomycin X4457 | Uterine Strip | 4,000 = 0.10 |
| Viomycin X4457 | Cat Depressor | 10,200 = 0.10 ± 5.54% |
| Viomycin X4457 | Dog Depressor | 2,630 = 0.10 ± 9.48% |
| Viomycin X4457 | Dog Depressor | 2,700 = 0.10 |
| Chloromycetin Ferm. | Dog Depressor | Much >4,000 = 0.10 |
| Chloromycetin Ferm. | Cat Depressor | 16,000 = 0.10 |
| Chloromycetin Synth. | Dog Depressor | Much >4,000 = 0.10 |
| Chloromycetin Synth. | Cat Depressor | Much >4,000 = 0.10 |
| Chloromycetin X5457 | Dog Depressor | About 3,000 = 0.10 |
| Chloromycetin X5457 | Cat Depressor | About 16,000 = 0.10 |

TABLE III.—EFFECTS OF HISTAMINASE ON THE HISTAMINE-LIKE ACTIVITY OF ANTIBIOTICS

| Product | Treatment | Method | Results |
|---|---|---|---|
| Histamine | Diamine oxidase, 1 hr. | Cat | Some reduction. 33 mm. to 27 |
| Histamine | Diamine oxidase, 24 hr. | Cat | Reduced to 20%. Equal response with 5× dose |
| Histamine | Diamine oxidase, 5 days | Cat | Reduced to 4%. Equal response with 25× dose |
| Penicillin and dihydrostrep. | Before diamine oxidase | Cat | 6,000 μg. = 0.10 μg. Hist. Base |
| Penicillin and dihydrostrep. | Diamine oxidase, 5 days | Cat | 6,670 μg. = 0.10 μg. Hist. Base |
| Penicillin and dihydrostrep. | Before diamine oxidase | Dog | 9,627 μg. = 0.10 μg. Hist. Base |
| Penicillin and dihydrostrep. | Diamine oxidase, 5 days | Dog | 6,800 μg. = 0.10 μg. Hist. Base |
| Dihydrostrep. | Before diamine oxidase | Cat | 33,970 μg. = 0.10 μg. Hist. Base |
| Dihydrostrep. | Diamine oxidase, 5 days | Cat | 17,000 μg. = 0.10 μg. Hist. Base |
| Dihydrostrep. | Before diamine oxidase | Dog | 10,836 μg. = 0.10 μg. Hist. Base |
| Dihydrostrep. | Diamine oxidase, 5 days | Dog | 8,900 μg. = 0.10 μg. Hist. Base |

of the data indicate that the histamine-like activity of the antibiotics treated is not due to the presence of histamine itself.

Table IV shows the very erratic results obtainable with either the dog or the cat depressor method for histamine-like activity from 25% solutions of Chloromycetin in 50% N,N-dimethylacetamide. The solvent alone shows a histamine-like activity of about 1 part in 100,000, and 0.5 cc. in five equal intravenous doses given within an hour was sufficient to kill a cat. Dogs survived larger doses but in many cases their subsequent reactions to histamine itself was greatly reduced. Of course, for therapeutic use this product is diluted at least 100 times and has never produced symptoms of any kind where the dose per Kg. of body weight is so very much smaller than that used in these animal tests. Since Chloromycetin itself shows no appreciable histamine-like action, it should not be necessary to test this special solution for histamine-like activity.

## DISCUSSION

It is apparent from the data submitted that dogs are as satisfactory as cats in the assay for histamine-like activity in some antibiotic preparations. While cats are somewhat more sensitive to histamine and

TABLE IV.—EFFECT OF N,N-DIMETHYLACETAMIDE ON THE HISTAMINE-LIKE ACTIVITY OF CHLOROMYCETIN

| Product | Activity in 50% Solvent μg. | Hist. Base | Activity in 1% Solvent μg. | Hist. Base | Animal | Response to Histamine, Mm. Before and After | |
|---|---|---|---|---|---|---|---|
| 160564 | 10,000 | = 0.10 | 7,500 | = 0.10 | Dog | 21 | 22 |
| 160564 | 16,700 | = 0.10 | 12,000 | = 0.10 | Cat | 19 | 10 |
| X4068 | 20,000 | = 0.10 | 6,000 | = 0.10 | Dog | 32 | 37 |
| X4068 | 25,000 | = 0.10 | 6,670 | = 0.10 | Cat | 18 | 17 |
| 160566 | 10,000 | = 0.10 | 5,000 | = 0.10 | Dog | 15 | 5 |
| 160566 | 29,400 | = 0.10 | ... | | Cat | 18.6 | 9 |
| 160566 | 10,000 | = 0.10 | ... | | Cat | 24 | 28 |
| X4067 | 18,000 | = 0.10 | ... | | Dog | 27 | 26 |
| X4067 | ... | | 24,000 | = 0.10 | Cat | 19 | 19 |
| 162076 | 8,700 | = 0.10 | 10,000 | = 0.10 | Dog | 10 | 3 |
| 160570 | 6,670 | = 0.10 | 20,000 | = 0.10 | Dog | 22 | 13 |

Solvent alone. 0.1 cc. given 5 times averaged 60 mm. and killed the cat. 0.0006 mg. Histamine Av. 40 mm.

Solvent alone. 0.1 cc. given 2 times averaged 18 mm. Dog 0.0005 mg. Histamine Av. 18 mm.

Solvent alone. 0.1 cc. given 7 times averaged 4 mm. Dog 0.0005 mg. Histamine Av. 7 mm.

1:1,000,000 solutions may ordinarily be used, a 1:200,000 solution must be employed for dogs, but the slope of their response to graded doses is as steep as that given by cats. The standard error when either animal is used is about ±20%; however this is satisfactory for a safety test which is intended only to show the relative absence of trace contaminants with histamine-like action.

Some highly purified antibiotics such as penicillin and Chloromycetin have shown enough freedom from appreciable histamine-like activity in these depressor tests that routine submission to such safety test should be discontinued. Streptomycin and viomycin, on the other hand, have displayed sufficient evidence of histamine-like activity to warrant continuation of such a safety test.

The use of antihistaminics such as Benadryl and Ambodryl to antagonize or inhibit histamine or histamine-like activity in some antibiotics is not feasible. Many tests have shown that after the first injection or two of such antihistaminics in intravenous doses as small as 1 or 2 mg., the sensitivity of both cats and dogs to histamine and to histamine-like activity is so decreased as to make them unsatisfactory for further use.

Histaminase (diamine oxidase) (7) was not very effective in its action on the histamine-like activity of some antibiotics. It was shown that histamine itself in a 1:200,000 solution was reduced by treatment with diamine oxidase for five days to 4% of this amount or 1:5,000,000. However, the traces of histamine-like activity in streptomycin, for instance, were erratically affected, and the presence of the histaminase in such solutions apparently added another variable factor.

The assay of Chloromycetin (25%) in a special solvent (50% N,N-dimethylacetamide) was also unsatisfactory. The solvent itself was chiefly responsible for production of an appreciable depressor reaction if the undiluted solution was administered to the animals intravenously, and even produced toxic symptoms from repeated dosages in a small animal like the cat. When diluted, the solution gave practically no depressor reaction, indicating that such a safety test on this preparation is entirely unnecessary. The standard error of such an assay would be fully ±100%.

## SUMMARY AND CONCLUSIONS

1. Dogs are satisfactory for use in the assay of histamine-like activity of some antibiotics, since the slope of their response to graded doses is as steep as that of cats.

2. Chemically pure antibiotics such as crystalline penicillin and Chloromycetin do not require a safety test for histamine but others such as streptomycin and viomycin do.

3. Antihistaminics and histaminase are of very limited value in the assay of antibiotics for histamine or for differentiating between true histamine and histamine-like activity.

4. Chloromycetin solution in N,N,-dimethylacetamide cannot be satisfactorily assayed for histamine-like activity, and such an assay should not be necessary for this preparation of a crystalline antibiotic.

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# Notes

## A Note on Solid Brominated Agents as *in Vitro* Bacteriostatics in Ointment Base Vehicles*

By PHILIP C. MERKER† and JOSEPH A. VONA‡

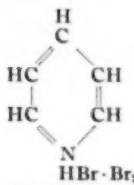
THREE solid type brominated reagents,[1] pyridinium bromide perbromide, N-bromosuccinimide, and tri-N-bromomelamine have been incorporated with Carbowax 1500[2] and white ointment U. S. P. XIII. The relative *in vitro* germicidal activities and stability of these preparations have been tested against *E. coli* and *Micrococcus pyogenes* var. *aureus*. The practical pharmaceutical use of free elemental bromine has been immeasurably lessened because of its annoying fumes, high oxidative powers, and danger of handling and storage.

With the advent of solid type brominated compounds, such as tri-N-bromomelamine (TNBM), pyridinium bromide perbromide (PBPB), and N-bromosuccinimide (NBS), the possibility of utilizing the bactericidal activity of bromine is facilitated. The latter two "solid bromine" compounds have been generally successful as sources of bromine for organic synthesis (1–3). The utilization of these compounds in suitable ointment bases with subsequent *in vitro* bacteriostatic effects is demonstrated in the experimental section.

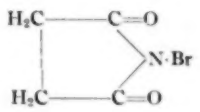
### EXPERIMENTAL

Pyridinium bromide perbromide (pyridine hydrobromide perbromide) $C_5H_6NBr \cdot Br_2$ is a red, crystalline, stable salt, m. p. 135–136° (dec.) with previous softening.

N-bromosuccinimide, $C_4H_4NBr$, is a white salt, m. p. 193–195°.

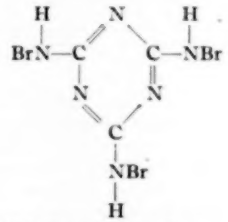


Pyridinium bromide perbromide (PBPB)



N-Bromosuccinimide (NBS)

Tri-N-bromomelamine, $C_3H_3N_6Br_3$, is an orange powder which sublimes at 170–172°.



Tri-N-bromomelamine (TNBM)

Two series of 5% (w/w) ointments were prepared. One series utilized Carbowax 1500 as a vehicle. The second series utilized white ointment, U. S. P. XIII, as the base.

Five grams of reagent was levigated with light liquid petrolatum (2.0 ml.). This mixture was then incorporated with sufficient base to make 100 Gm. of finished product.

The test organisms consisted of *E. coli*[3] and *Micrococcus pyogenes* var. *aureus*.[4] The experimental plates were seeded with 0.1 ml. of a well-mixed twenty-four-hour broth culture. Ten milliliters of fused nutrient agar at 45° was then poured over the culture, and the mixture was agitated and then permitted to solidify.

One-half gram of 5% brominated ointment, encased in 20-mm. single-shell aluminum seals, was placed in the center of each experimental Petri dish. Two additional plates were run with each test organism. One plate, the blank, consisted of 0.5 Gm. of plain ointment base encased in the aluminum seal, plus nutrient agar and the test organism. The second plate, the control, contained nutrient agar and the test organism alone.

All plates were incubated at 37° and observations were made at twenty-four and forty-eight-hour intervals. The procedure was repeated three times. The first test was conducted immediately after the ointments were made, the second test after the ointments were one week old, and the third test after they were two weeks old. During the intervening days of the experiment, the ointments were kept at 25° in dark green, tightly-closed glass containers with plastic tops.

* Received July 6, 1951, from the Department of Chemistry, Brooklyn College of Pharmacy, Brooklyn, N. Y.

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‡ Thanks are due to Professor Alexis V. Pfeiffer, Department of Microbiology, for conducting the bacteriological tests.

[1] Pyridinium bromide was supplied by Jasons Drug Co., Brooklyn, N. Y.; N-bromosuccinimide by Arapahoe Chemicals, Boulder, Colo.; and tri-N-bromomelamine by Wallace and Tiernan, Belleville, N. Y.

[2] The generous supplies of Carbowax 1500 were supplied by Carbide & Carbon Chemical Corp., New York, N. Y., and the aluminum seals by Lederle Laboratories, Pearl River, N. Y.

[3] *Escherichia coli* No. 9492, American Type Culture Collection, Brooklyn College, Brooklyn, N. Y.

[4] *Micrococcus pyogenes* var. *aureus* No. 6538, American Type Culture Collection, Brooklyn College, Brooklyn, N. Y.

TABLE I.—IN VITRO GERMICIDAL EFFECTIVENESS OF 5% PREPARATIONS AGAINST E. COLI

| | Freshly Prepared | | 7-Day Preparation | | 14-Day Preparation | |
|---|---|---|---|---|---|---|
| | 24 Hr. | 48 Hr. | 24 Hr. | 48 Hr. | 24 Hr. | 48 Hr. |
| **White Ointment** | | | | | | |
| Blank[b] | + | + | + | + | + | + |
| NBS | + | + | + | + | 1 mm. | 1 mm. |
| PBPB | + | + | 1 mm. to 0.5 cm. | 2 mm. to 1.5 cm. | 3 mm. | 5 mm. |
| TNBM | + | + | + | + | + | + |
| Control[c] | + | + | + | + | + | + |
| **Carbowax 1500[a]** | | | | | | |
| Blank[b] | 3 mm. | 4 mm. | 1 mm. to 1 cm. | 1 mm. to 1 cm. | 1.5 mm. to 2 mm. | 0.5 mm. to 2.5 mm. |
| NBS | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| PBPB | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| TNBM | 1 cm. to 3 cm. | 1 cm. to 3 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| Control[c] | + | + | + | + | + | + |

[a] All carbowax preparations indicate complete fusion at 37°.
[b] Blank equals plain ointment plus test organism and nutrient agar.
[c] Control equals test organism and nutrient agar.

## DISCUSSION

The general *in vitro* effectiveness of the reagents pyridinium bromide perbromide, tri-N-bromomelamine, and N-bromosuccinimide when incorporated into Carbowax 1500 against *E. coli* and *Micrococcus pyogenes* var. *aureus* is illustrated in Tables I and II.

The activity of these brominated reagents in Carbowax 1500 against the test organisms manifested itself by yielding zones of inhibition greater than 7 cm. The only time that a zone of inhibition less than 7 cm. was obtained occurred when the fresh preparation of tri-N-bromomelamine was tested.

The Carbowax preparations of these reagents showed complete stability during the test period. None of the ointments developed "ester-like" odors or darkened in color, and none developed white acrid fumes.

The *in vitro* bacteriostatic activity against *Micrococcus pyogenes* var. *aureus* of the fresh preparation of N-bromosuccinimide in white ointment expressed itself as a well defined 4-mm. zone of inhibition after twenty-four hours. After the ointment was fourteen days old, a zone of inhibition of 6 mm. was obtained although the ointment base had broken down; the preparation darkened in color and developed an "ester-like" odor. No white acrid fumes were produced. This same reagent, in white ointment, displayed slight activity against *E. coli* consisting of a 1-mm. zone of inhibition produced within twenty-four hours by the fourteen-day-old preparation.

Pyridinium bromide perbromide in white ointment indicated *in vitro* activity of an ascending type against *Micrococcus pyogenes* var. *aureus*, although this ointment preparation was the least stable of the series. There was darkening and development of white acrid fumes within seven days. These fumes indicate the possible formation of HBr due to the interaction of the released bromine of the reagent with the alcohols and fatty acids present in the white ointment base. The freshly prepared perbromide in the white ointment vehicle showed no *in vitro* activity against *E. coli*. After seven days, readable zones were obtained but the ointment had broken down completely, as indicated by darkening in color and development of white acrid fumes.

The least active member of the series was tri-N-bromomelamine. No activity was displayed by this reagent against the organisms during the entire period of *in vitro* testing when white ointment was

TABLE II.—IN VITRO GERMICIDAL EFFECTIVENESS OF 5% PREPARATIONS AGAINST MICROCOCCUS PYROGENES. VAR. AUREUS

| | Freshly Prepared | | 7-Day Preparation | | 14-Day Preparation | |
|---|---|---|---|---|---|---|
| | 24 Hr. | 48 Hr. | 24 Hr. | 48 Hr. | 24 Hr. | 48 Hr. |
| **White Ointment** | | | | | | |
| Blank | + | + | + | + | + | + |
| NBS | 4 mm. | 5 mm. | 5 mm. | 5 mm. | 6 mm. | 6 mm. |
| PBPB | 1 mm. | 1 mm. | 6 mm. | 6 mm. | 10 mm. | 8 mm. |
| TNBM | + | + | + | + | + | + |
| Control | + | + | + | + | + | + |
| **Carbowax 1500** | | | | | | |
| Blank | 1 mm. | + | 1 mm. to 5 mm. | ... | 2 mm. | 2 mm. to 2.5 mm. |
| NBS | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| PBPB | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| TNBM | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. | >7 cm. |
| Control | + | + | + | + | + | + |

used as a vehicle. However, this preparation showed the greatest amount of stability.

## CONCLUSION

The *in vitro* germicidal activity of pyridinium bromide perbromide, N-bromosuccinimide, and tri-N-bromomelamine, in a 5% concentration when incorporated with white ointment U. S. P. XIII, is less than a 5% ointment preparation with Carbowax 1500 as the sole vehicle. The stability of white ointment, U. S. P. XIII, when used as a base for pyridinium bromide perbromide, N-bromosuccinimide, and tri-N-bromomelamine yields the following series in which tri-N-bromomelamine produces the most stable preparation:

Tri-N-bromomelamine > N-bromosuccinimide > pyridinium bromide perbromide.

Carbowax 1500 is a more suitable ointment vehicle for the three brominated compounds than is white ointment, U. S. P. XIII.

Toxicity and sensitivity tests were not carried out with any of the preceding preparations. These tests must be carried out before the preparations can be used on humans.

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# A Note on Experimental Polycythemia Produced by Phenylephrine Hydrochloride*

By JOHN EMERSON DAVIS

POLYCYTHEMIA has been produced in various animals by the daily administration of such vasoconstrictor drugs as epinephrine and posterior pituitary injection (1), and ephedrine and benzedrine (2). It has also been induced in man by ephedrine (2, 3) and benzedrine (4). The polycythemias produced by some of these drugs have been abolished or reduced by the additional administration of raw liver or vasodilator drugs (5, 6), or daily inhalation of pure oxygen (7), as has also the polycythemia induced by cobalt or by exposure to low atmospheric pressure (5, 6).

Since phenylephrine is a long-acting vasoconstrictor drug which does not manifest tachyphylaxis, it was decided to investigate whether this drug could produce a polycythemia.

## PROCEDURE

Six adult rabbits were placed on a constant adequate diet and allowed water *ad lib.* Erythrocyte counts were made on blood obtained periodically from the marginal ear veins until the normal or control value was established for each rabbit. The animals were then given daily subcutaneous injections of 1.5 mg. of phenylephrine hydrochloride. After polycythemia had appeared and had been maintained for a few weeks, the drug administration was discontinued and the recovery of the animals toward normal was studied. It should be emphasized that blood samples always were taken at least twenty hours after the preceding daily injection of Neosynephrine, since only the chronic changes in erythrocyte counts were studied.

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Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

## RESULTS

The erythrocyte numbers of all animals showed significant increases within five to eighteen days after the commencement of phenylephrine medication (see Fig. 1). The polycythemias persisted throughout the periods of drug administration, which ranged from twenty-four to thirty-six days. The maximum increases in red cell counts ranged from 12.5 to 27.5% with an average of 17%. After cessation of phenylephrine administration, about fifteen days was required for the erythrocyte numbers to return to their normal pre-experimental values.

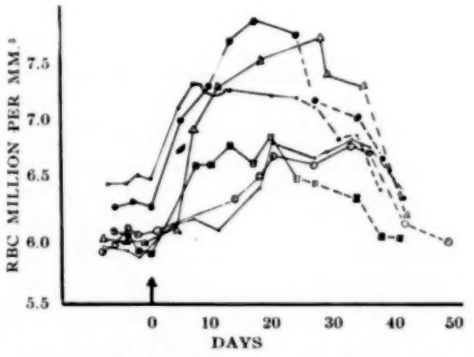


Fig. 1.—The production of polycythemia in six rabbits by the daily subcutaneous injection of 1.5 mg. of phenylephrine hydrochloride. Dashed lines indicate discontinuation of drug administration for each animal.

## DISCUSSION

It is believed that the polycythemias obtained in these rabbits represent true increases in the red cell counts of the animals, mainly because of the slow development of the increases and the gradual return to normal after discontinuation of medication. In previous work with epinephrine and ephedrine (1, 2), this same slow development of, and recovery from, polycythemia was observed. A mild reticulocytosis was also found to occur early in the course of medication, and it was noted that total leucocyte counts remained unchanged before, during, and after medication. Furthermore, the polycythemia was produced in splenectomized as well as normal animals (1, 2).

That relatively unopposed activity of the sympathetic vasoconstrictor nerves can produce polycythemia has been shown by Schafer (8), who surgically interrupted the carotid sinus and aortic arch depressor reflexes in dogs. A high percentage of these animals developed neurogenic hypertension and some of them acquired a true polycythemia.

It has been demonstrated that acetylcholine administration can produce anemia in dogs (9), and Fornaroli and Antona (10) have produced the same effect in rabbits and rats.

These facts suggest strongly that the autonomic nervous system, together with its neurohormones, probably exerts a degree of regulatory control over the rate of erythropoiesis during health, which may become unbalanced in disease.

## SUMMARY

The daily subcutaneous administration of 1.5 mg. of phenylephrine hydrochloride to six rabbits caused the development of polycythemia in all within five to eighteen days. Maximum increases in red cell counts ranged from 12.5 to 27.5 per cent, an average of 17 per cent. After cessation of drug administration, a period of about fifteen days was required for the erythrocyte numbers to return to their normal pre-experimental values.

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## BOOKS RECEIVED

*Commercial Directory of Africa, 1950–1951.* International Marketing Service Corp., Nairobi, Kenya Colony, Africa, 1951. xxiv + 279 pp. 24.5 x 16 cm. Price $5.00.

*Accepted Dental Remedies 1953.* 18th ed. Council on Dental Therapeutics, American Dental Association, Chicago, Illinois, 1952. xvi + 208 pp. 20.5 x 16 cm. Price $2.00.

*Non-Aqueous Solvents.* By LUDWIG F. AUDRIETH and JACOB KLEINBERG. John Wiley & Sons, Inc., New York, 1953. xii + 284 pp. 15.5 x 23.5 cm. Illustrated. Price $6.75.

*Methoden der organischen Chemie.* Band VIII. Herausgegeben von EUGEN MÜLLER. Georg Thieme Verlag, Stuttgart, Germany, 1952. xviii + 775 pp. 18 x 26.5 cm. Price DM 98.

*Comptes Rendus—IV^es Journees Pharmaceutiques Nationales. Premier Symposium International sur le Controle des Specialites Pharmaceutiques.* Association Pharmaceutique Belge, 1952. 435 pp. 16 x 24 cm.

*1952 Year Book of Drug Therapy.* HARRY BECKMAN, Editor. The Year Book Publishers, Inc., Chicago, 1952. 606 pp. 13.5 x 20 cm. Illustrated. Price $5.50.

*Side Effects of Drugs.* By L. MEYLER. Trans. by PH. VUIJSJE and W. MULHALL CORBET. Elsevier Press, Inc., Houston, Texas, 1952. xii + 268 pp. 15.5 x 23 pp. Price $5.50.

*Chemical Control of Insects.* By T. F. WEST, J. ELIOT HARDY, and J. H. FORD. John Wiley & Sons, Inc., New York, 1952. xi + 211 pp. 14 x 19 cm. Price $3.25.

*Neomycin.* By SELMAN A. WAKSMAN. Rutgers University Press, New Brunswick, N. J., 1953. xiv + 219 pp. 16 x 23.5 cm. Price $4.00.

*University of California Hospital Formulary and Compendium of Useful Information.* 2nd ed. Compiled by the Hospital Pharmacy Committee of the University of California Medical Center. University of California Press, Berkeley and Los Angeles, 1952. 318 pp. 10 x 17 cm. Price $3.75.

# Book Notices

*Quantitative Analysis: Elementary Principles and Practice.* By HARVEY DIEHL and G. FREDERICK SMITH. John Wiley & Sons, Inc., New York, 1952. vii + 539 pp. 15.5 x 24 cm. Illustrated. Price $5.00.

This is not just another elementary textbook on quantitative analysis. The authors, Harvey Diehl, Professor of Chemistry at Iowa State College, and G. Frederick Smith, Professor of Chemistry at the University of Illinois, have presented the subject in a refreshing manner which should stimulate student interest.

In the first four chapters the nature and general operations of quantitative analysis, the use of the balance, and the calculations involved in gravimetric analysis are presented in an unusually clear style. The orientation to quantitative analysis in these chapters is of a quality seldom equaled in an elementary textbook on this subject.

In the preface, the authors state that qualitative analysis as a separate course is disappearing from the curricula of American colleges and its position in general chemistry courses is shrinking. Because of this, their *Quantitative Analysis* is directed toward the sophomore level. Theory begins with the solubility principle, and calculations in the beginning presuppose a background only in general chemistry. The subject matter is arranged so that gravimetric analysis precedes volumetric analysis, which the authors and many others consider a more rational approach.

Following the introduction to gravimetric and volumetric procedures is a treatment of neutralization and oxidation-reduction, each of which is covered by a chapter on theory and one on practice. Volumetric reactions involving the formation of insoluble or nondissociated compounds are adequately treated in a separate chapter.

The book also includes an excellent section on colorimetry, and the determination of *p*H by both colorimetric and potentiometric methods characterized by unusual clarity of presentation, which adds materially to the completeness of the volume by explaining the application of these important analytical procedures.

The traditional analyses of brass and of limestone are, of course, also fully treated, and there are chapters on methods of separation, electrodeposition, and errors. Another attractive feature of *Quantitative Analysis* is an appendix which contains useful tables, a glossary of terms used in analytical parlance, and a 5-place logarithm table. There are more than 100 illustrations, a high proportion reproduced from line drawings, which supplement and emphasize the material discussed in the text. The printing and binding are of good quality and result in an attractive book. An adequate index is provided.

Another desirable feature of the volume is the inclusion of more than 450 problems which have been well chosen to illustrate methods of analysis and their application to the analysis of products not covered in the general treatment.

*Quantitative Analysis* strikes a good balance between theory and practice. It deserves wide acceptance as an introductory textbook.

*Statistics of Bioassay.* By C. I. BLISS. Academic Press Inc., New York, 1952. iii + 628 pp. 15.5 x 23.5 cm. Price $3.50.

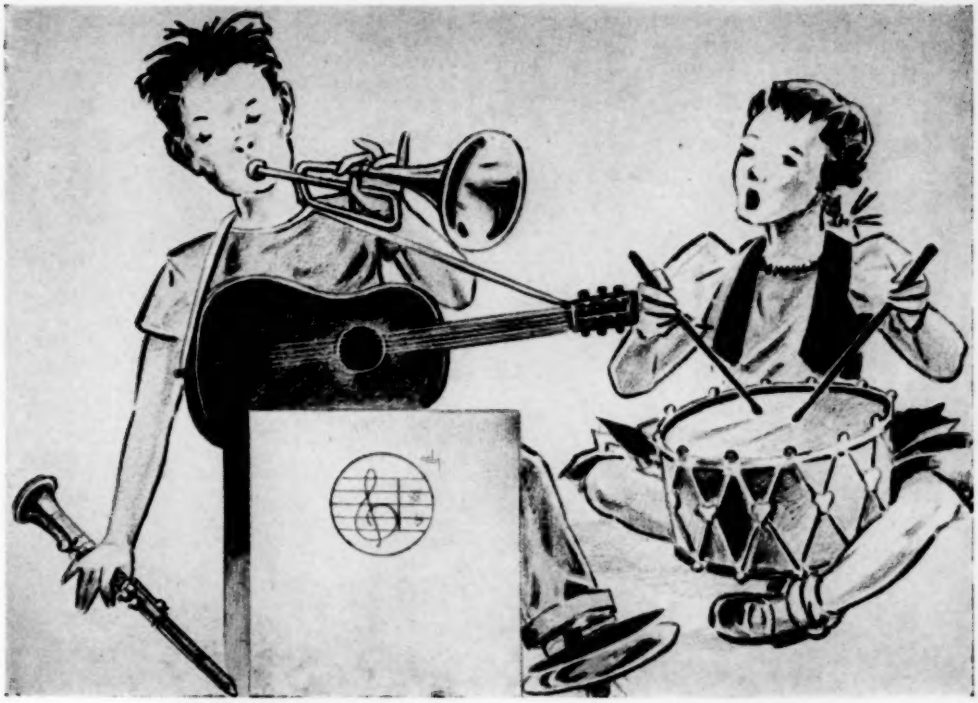
The basic material in this book was first published as the chapter on "Statistical Methods in Vitamin Research" in the book *Vitamin Methods. Vol. II*, which was reviewed in THIS JOURNAL, **40,** 639(1951). Although the text stresses the applications of statistical methods to vitamin assays, it is an effective description of the procedures common to all areas of biological assay.

*The Extra Pharmacopoeia (Martindale).* Vol. I. 23rd ed. The Pharmaceutical Press, London, 1952. xxii + 1,352 pp. 12.5 x 19 cm. Price 55*s*.

It is the opinion of Dr. K. R. Capper, editor of the scientific publications of the Pharmaceutical Society of Great Britian, that this book in its 23rd edition and seventieth year is so popular with pharmacists and medical practitioners because it "has provided the type of information which the users require." Much of the subject matter is presented in a manner similar to that of the U. S. Dispensatory, also in its 23rd edition, although more therapeutic and medical data are included in this British publication. Differences in standards and dosages of the same drug in different pharmacopeias are indicated.

Much useful information is given in special sections of the book; 100 pages are devoted to a section on antibiotics and 35 pages are used for a therapeutic and pharmacological index. Throughout the text, drugs are listed by official names (B. P.) or other generic names, e. g., Methapyrilene Hydrochloride (N. N. R.). Synonyms and proprietary names are included.

Dr. Capper, in an address on *The Extra Pharmacopeia*, stated: "Although we consider that every encouragement should be given to the use of Approved Names, the *Extra Pharmacopoeia* would lose its value as a book of information if proprietary names were not also given. But to meet the criticism that such names were given too much prominence in previous editions, we have put them in small type, which, we understand from some reviewers, makes them almost invisible to the less keen-sighted. This will enable those who disapprove of such names to develop a temporary ocular deficiency and so avoid seeing them." Unfortunately, this witty explanation might apply to much more of the text, and the use of such small type evokes this reviewer's only complaint about a splendid reference volume. No pharmaceutical or medical library should be without a copy of *The Extra Pharmacopoeia* of Martindale.



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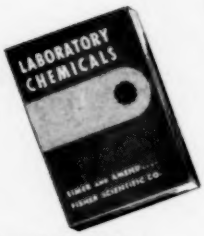


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